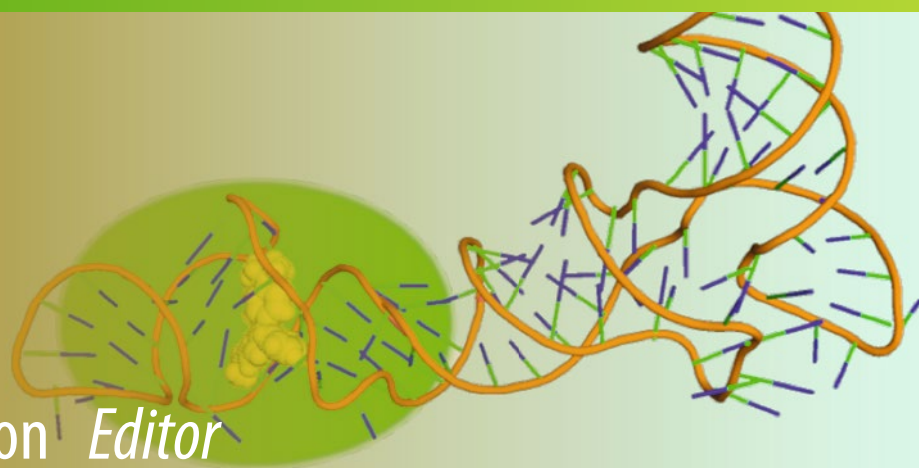


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Luc Ponchon *Editor*

RNA Scaffolds

Methods and Protocols

 Humana Press

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RNA Scaffolds

Methods and Protocols

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 **Humana Press**

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Preface

RNAs are at the center of numerous cellular phenomena and play very different roles in each. One of their roles is in particular that of organization center: the ribosome, RNA telomerase, and “Long Noncoding RNAs” are, among others, examples of RNA structures that recruit other molecules and organize biological processes. These RNAs possess structures allowing interactions with other molecules (proteins, ligands) and thus will potentialize molecular reactions.

Advances in structural biology have permitted a definition of the rules with regard to the folding of RNA allowing us today to better understand exactly how they fold and interact. As opposed to DNA, RNAs are able to adopt very variable folds and therefore are able to adopt ligand-specific structures. Contrary to proteins, we are able to create structures composed of different “RNA” modules, each of which is able to keep its activity independent from the others.

So, when they are stable, folded RNA can be used as a tool for biological, pharmacological, and/or molecular design studies. RNA presents the peculiarity, like Meccano, of being able to fold into structural domains, which can assemble and sometimes form supra-molecular objects. We can isolate, modify, or create an RNA template *de novo* to make use of its recognition or enzymatic functions.

From my point of view, an “RNA scaffold” is a synthetic or natural RNA whose structure, for example, allows one to optimize a reaction, to isolate a molecule, or to favor an interaction.

Like Biobricks, the tools based on RNA scaffolds are an example of the emergence of synthetic biology. Indeed, they participate in the creation and construction of biological objects and systems for useful purposes.

In this volume, we have tried to be as representative as possible of that which is done today. You will find detailed here processes and techniques that differ greatly from one to another.

This book reviews recently developed techniques that use “RNA scaffolds” as molecular tools. These methods cover domains as various as molecular biology, cellular biology, nanotechnology, and structural biology.

Contents

In order to design a scaffold, it is sometimes necessary to possess certain structural data. A structure can be modeled from an RNA structure data bank. In the first chapter, Chen and his colleagues describe their prediction method, Vfold, that, from a primary sequence, allows one to determine a three-dimensional model of an RNA. This method is based on a pattern-based approach. Thus, they extract different patterns (such as hairpin loops, internal loops, pseudoknot loops, and three-way junctions) from the two-dimensional structure. Based on this data, the implemented algorithm calculates a three-dimensional model.

However, if one seeks to obtain an RNA structure at an atomic level (e.g., for the rationalized design of a scaffold), crystallography is the technique of choice. RNA crystallogensis

remains nonetheless complicated. Indeed, RNAs, as opposed to proteins, do not crystallize as easily and the X-ray diffraction is often weak. In Chapter 2, Ferré d'Amaré and his colleagues describe techniques allowing an improvement in data acquisition and notably for large RNA (over 100 nucleotides) for which only very few structures exist.

They propose a protocol allowing the increase in diffraction power of the crystals by combining two techniques. The first consisting of dehydrating the crystal, the second of substituting the divalent ions with strontium ions. Through the example of a gene-regulatory tRNA-mRNA complex, they show us the importance of these two techniques on the quality of diffraction.

A limiting factor for RNA study (notably for long RNAs) is obtaining them in large quantities. Three classic techniques exist in order to obtain the RNA: in vitro transcription, chemical synthesis, and cellular extraction.

The three following chapters describe the techniques permitting, as with proteins, the in vivo production of RNA. Chapter 3 describes a system of RNA protein co-production in the bacteria. This protocol is the logical progression of protocols already described in a previous book *Recombinant and In Vitro RNA Synthesis* in the same collection. In the aforementioned book, the authors describe the production of RNA protected from bacterial nuclease by a “tRNA” camouflage. The RNA is protected at its extremities by the tRNA chassis and accumulated in the bacteria. In this protocol, they propose to co-produce RNA-protein complexes and thus show the advantage of having a joint production of the two molecules. In Chapter 4, Wijmenga and his colleagues also use the tRNA camouflage but have added two ribozymes to the extremities of the RNA in question. Through cleaving, they can thus recuperate their RNA separated from the tRNA chassis. In their protocol, they present a labelled RNA for NMR study.

Fox and his colleagues offer a method also permitting the overproduction of RNA in bacteria in Chapter 5. They use a different chassis: the RNA ribosomal 5S. Indeed, like the tRNA, the RNA 5S possesses a fold that allows it to be accumulated in the bacteria. Their protocol thus goes into detail on the production of different RNA and how to make the cleave. So as to liberate the RNA of interest from the 5S, they propose a cleavage via the DNazyme. These short sequences of DNA hybridize themselves with the RNA to form a structure of ribozyme-like and thus allow the RNA cleavage.

Affinity or “pull-down” techniques allow the identification of molecular complexes. In these techniques, a protein linked to a matrix serves as bait. From the cellular extracts, one can isolate the linked molecules. Macara and his colleagues propose a pull-down system in which RNA serves as bait in order to identify the RNA-binding proteins. To render the system specific and robust, their RNA bait is embedded in a tRNA scaffold and possesses an aptamer for the streptavidin in order to isolate RNA-protein complexes from extracts of mammalian cells.

Fluorescence remains a technique of choice to perform cellular localization. Green fluorescent protein (GFP) and its numerous derivatives allow one to localize the proteins in cells or tissues. However, it remains difficult to specifically locate the RNA as no naturally fluorescent RNA has been identified to date. It is therefore necessary to use indirect techniques. The two following chapters describe RNA scaffolds allowing one to make cellular localization of RNA or metabolites through fluorescence.

Broude and his colleagues describe to us in Chapter 7 a technique permitting the identification of the presence of an RNA in cells. The scaffold is composed of an antisense RNA, an RNA target, and two RNA aptamers. When the antisense hybridizes itself with the RNA target, the two aptamers can recruit two fusion proteins, which will contemplate each other

and then form the GFP and therefore fluoresce. It is therefore via the fluorescence of the GFP that they localize their RNA.

In Chapter 8, Hammond and his colleagues propose to us a technique allowing the localization of a metabolite via an RNA scaffold, which is linked to a fluorescent Chromophore. This RNA is a biosensor composed, on the one hand, of a “spinach aptamer” which links a fluorescent chromophore to DFHB1 and, on the other hand, of a specific riboswitch of the cyclic di-GMP. The idea being that the fixation of the fluorophore derives from the fixation of the di-GMP to its aptamer.

In order to observe the intracellular phenomena, it is sometimes necessary to optimize the phenomena for it to be observable. “Zhu China team” is a team of students who have participated, like many other student teams, in the IGEN 2012 competition. This team, under the guidance of their tutor, describes to us a protocol allowing one to potentialize the meeting of two proteins in a cell. Lindner, Silver, and their colleagues developed this method. It concerns an RNA composed of two aptamers, which recruit two proteins and therefore force their meeting. The Zhu China team describes to us in Chapter 9 how they have made the interaction dependent of a sensor. It is the fixation of the theophylline to the sensor that renders the RNA accessible to the proteins and allows thus their coming together and their interaction.

RNA in vitro evolution or SELEX enables the artificial evolution and selection of RNA molecules that possess a desired property, such as binding affinity for a particular ligand or an activity such as that of an enzyme or catalyst. The first such selections involved isolation of various aptamers that bind to small molecules. The first catalytic RNAs produced by in vitro evolution were RNA ligases, catalytic RNAs that join two RNA fragments to produce a single adduct.

In Chapter 10, Ikawa and his colleagues describe how they have created a DSL ribozyme (designed and selected ligase). They show us how these systems allow the reduction of the number of random sequences to screen and how these scaffolds of a well-defined structure ease the screening.

The two following chapters (11 and 12) describe two methods allowing the identification of riboswitches from a random bank of aptazymes (allosteric ribozymes). These systems are logic gates; indeed the aptazymes are composed of an aptamer RNA at a strategic position with regard to the self-cleaving ribozyme so that the structure of the ribozyme is stabilized or destabilized through the link of the ligand. These aptazymes are in the 5' position of an ARNm (coding for a reporter gene), which will or will not be degraded based on the efficiency of the riboswitch. Hartig and his colleagues have thus inserted random sequences of aptazymes in the 5'-UTR of the hRluc reporter gene on the plasmid psi-CHECK2 making the expression of the luciferase dependent on the ligand. As for Yokobayashi and his colleagues, they also work in the area of mammalian cells but use the reporter system EGFP. They describe a protocol allowing the screening, at a medium rate, around a hundred aptazymes directly into mammalian cells.

As we have just seen, certain RNAs can see their activity regulated by a ligand link like a small molecule or another RNA (in the case of aptazymes). The RNA can be used in nanotechnology like “programmable” polymers capable of assembling themselves and forming topologically complex structures. Jaramillo and his colleagues describe, in Chapter 13, a method allowing one to design scaffolds from small RNAs which stack and form nano-objects. Their protocol precisely describes how to model these objects *in silico* and the ways in which they can be characterized.

The flexibility and the versatility of the RNA allow them to adopt very varied structures. Many RNA, in particular, can assemble themselves in the form of nano-structures. We understand the determinants that govern the structure and the layout of these objects better and better. In the three following chapters, protocols allowing one to design and produce different RNA nano-structures are described. In Chapter 14, Saito and his colleagues show us how to design RNA origami. They thus manage to give their RNA a triangular structure. The angles are produced by the interaction of the L7Ae protein with recognition sequences placed in the RNA.

Guo and his colleagues have been working on the pRNA of phage phi29 for several years. Through matching bases, this RNA assembles itself naturally to form nano-structures. They have succeeded in demonstrating that this object could be functionalized and could thus have a therapeutic use.

In Chapter 16, Kumar and his colleagues offer us an original method to synthesize cadmium sulfide (cds) nanoparticles. During polymerization and formation of nanoparticles, the RNA will allow one to render the polymerization directional and optimize the interactions. More precisely, they describe to us how to form nano-objects in tube form. A whole series of techniques allowing the characterization of objects are described as a supplement.

The final chapter is devoted to an RNA scaffold that serves as a genetic tool. “Gene silencing” can be induced by small noncoding antisense RNA, which hybridizes on an RNA messenger preventing the gene translation. Silencing RNA (siRNAs) are thus small coding sequences allowing, in genetics, the switching off of a gene specifically and thereby understanding its function. In Chapter 17, Lee and his collaborators describe a method to us that allows the potentialization of the silencing activity of an siRNA. The siRNA is surrounded by two structures in a stem loop, which increase the half-life of the RNA and thus increase the silencing. This structure that they have named afsRNA (artificial small regulatory RNAs) could thus become a more efficient silencing tool.

In conclusion, I would like to thank all of the authors who have allowed this book to be published. Their work shows to what extent RNAs are fascinating and interest researchers in very different areas. Naturally, we are a long way from exploring their full potential and I hope that the reading of this book will inspire its readers, especially young researchers, to further study of this area.

Paris, France

Luc Ponchon

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Chapter 1

A Method to Predict the 3D Structure of an RNA Scaffold

Xiaojun Xu and Shi-Jie Chen

Abstract

The ever increasing discoveries of noncoding RNA functions draw a strong demand for RNA structure determination from the sequence. In recently years, computational studies for RNA structures, at both the two-dimensional and the three-dimensional levels, led to several highly promising new developments. In this chapter, we describe a recently developed RNA structure prediction method based on the virtual bond-based coarse-grained folding model (Vfold). The main emphasis in the Vfold method is placed on the loop entropy calculations, the treatment of noncanonical (mismatch) interactions and the 3D structure assembly from motif-based template library. As case studies, we use the glycine riboswitch and the G310-U376 domain of MLV RNA to illustrate the Vfold-based prediction of RNA 3D structures from the sequences.

Key words Partition function, Loop entropy, Mismatched stacks, 2D structure motif, Structure assembly

1 Introduction

To perform crucial cellular functions, RNA molecules fold up to form compact three-dimensional (3D) structures [1–5]. The RNA structure determination by experiments alone cannot keep up the pace with the ever increasing number of RNA sequences and new functions. The gap between the number of known RNA 3D structures and the number of biologically significant RNA sequences underscores more than ever the request for accurate computational models for RNA structure prediction.

An RNA structure can be described at the two-dimensional (2D) and three-dimensional (3D) levels. A 2D structure is defined as the sum of all the base-base pairs in the structure, including long-range base pairs in tertiary folds. Computational prediction of RNA 2D structures falls into two categories [6–10]: sequence comparison (alignment) analysis and free energy-based modeling. In general, sequence comparison-based methods can give more reliable predictions than free energy-based methods, but it depends on the availability of homologous sequences and often cannot directly provide information about the alternative structures.

For the free energy-based modeling, a key problem is to determine the helix stabilities and loop free energies. The free energy parameters for a helix stem can be calculated from the Turner experimental data [11], but the loop free energy requires a model.

The recently developed Vfold model is a statistical mechanics-based RNA folding model. The model relies on a coarse-grained (virtual bond) representation of RNA structures [12–14]. Compared with other free energy-based RNA 2D structure prediction models, such as Mfold [15] and RNAstructure [16], the Vfold model computes loop entropy parameters from explicit conformational sampling. Furthermore, by enumerating all the possible (sequence-dependent) intra-loop mismatches, the Vfold model partially accounts for the sequence-dependence of the loop free energy. Through application to a broad range of experimental and biological problems, the Vfold-based predictions have shown to be able to provide novel insights for RNA mechanisms, such as pseudoknot-involved conformational switch between bistable secondary structures [17], microRNA–gene target interactions [18], and RNA–RNA kissing dimerization in viral replication [19, 20].

Knowing RNA 2D structures alone is often not sufficient to understand RNA function. We also need RNA 3D structure information in order to understand the interactions between RNA and other molecules and RNA functions [21–24]. One way to predict RNA 3D structure is to combine a coarse-grained RNA structure model with the knowledge-based force field and fold the RNA through discrete molecular dynamics (DMD) simulations [25–28]. Due to the limitation of conformational sampling, this method would be most suitable for short RNAs or large RNAs with auxiliary constraints from experimental data. Based on the assumption that 3D structure is more conserved and can be recognized by the alignment of sequences and structure motifs, (3D structure) template-based modeling has become a promising method in RNA 3D structure predictions [29–31]. The template-based methods build RNA 3D structures using known structures ranging from fragments of 1–3 nucleotides to larger structural motifs. One of the common limitations for the template (structure assembly) approaches is the completeness of the fragment library. The lack of reliable structural motifs for many loops and junctions greatly hampers the success of accurate 3D structure prediction.

For a given 2D structure, the Vfold-based 3D structure prediction method searches for the appropriate template for each loop/junction in the structure, and assembles the 3D template structures into a scaffold for further structure refinement. In comparison with other template-based (structure assembly) methods such as FARNAL/FARFAR [29] and MC-Sym [31], which sample structures from small fragments of known RNA structures, the Vfold-based method uses motif-based instead of fragment-based templates.

2 Algorithms

2.1 RNA Motif-Based Loop Entropy

Using two virtual bonds per nucleotide to represent the backbone conformation, the Vfold model samples fluctuations of loops/junction conformations in 3D space through conformational enumeration (*see* Fig. 1). By calculating the probability of loop formation, it gives the conformational entropy parameters for the formation of the different types of loops such as hairpin, bulge, internal, pseudoknot loops. The model has the advantage of accounting for chain connectivity, exclude volume and the completeness of conformational ensemble.

1. Enumerate all the possible virtual bond backbone conformations for a given chain length (*see* **Note 1**) and count the total number Ω_{coil} of the conformations.
2. From the conformational ensemble above, identify the loop conformations according to the loop closure condition. For example, for hairpin loops, the two ends of a loop conformation should be fitted to an A-form base pair. Count the total number Ω_{loop} of loop conformations.
3. Calculate the loop entropy $\Delta S_{\text{loop}} = k_B \ln(\Omega_{\text{loop}} / \Omega_{\text{coil}})$. Here, k_B is the Boltzmann constant.

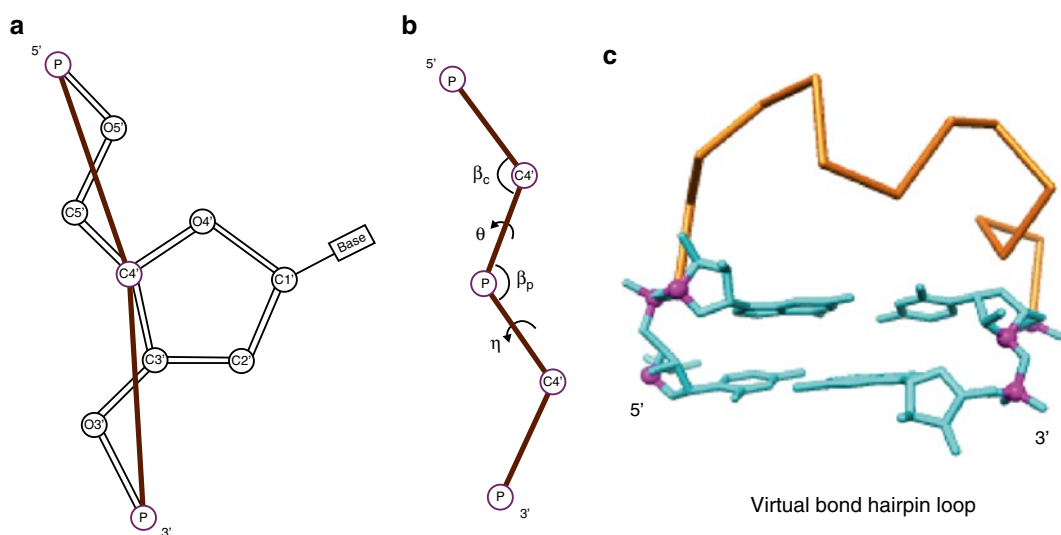


Fig. 1 Vfold computes loop entropies by sampling virtual bond conformations in 3D space. (a) Virtual bond representation: two bonds (P-C4' and C4'-P) per nucleotide. (b) The bond angles (β_c , β_p) and the torsional angles (θ , η) for the virtual bonds. Vfold enumerates RNA backbone conformations on a diamond lattice with bond length of 3.9 Å, bond angle of $\sim 109.5^\circ$ and three equiprobable torsional angles (60° , 180° , 300°). (c) A virtual bond backbone conformation of a hairpin loop, with two ends fitted to the base pair structure in an A-form helix

Table 1
RNA motif-based template library

Motif name	Number of templates
Hairpin loops	2,366
Internal/bulge loops	3,260
3-way junctions	820
4-way junctions	506
5-way junctions	222
6-way junctions	49
7-way junctions	61
H-type pseudoknots	56

4. Vfold computations lead to pre-tabulated entropy parameters for hairpin loops [12], internal/bulge loops [12], H-type pseudoknots with/without inter-helix junction [32, 33] and hairpin-hairpin kissing motifs [19].

2.2 RNA Motif-Based Template Library

The (3D structure) template library was built from 2,621 PDB structures (*see* **Note 2**), including RNA-involved complexes. It contains 3D templates for hairpin loops, internal/bulge loops, H-type pseudoknots, and multibranch junctions.

1. For a given RNA 3D structure, extract the A-form helices. From the information of helices and base pairs, the corresponding 2D structure is determined.
2. Identify all the non-helix 2D structure motifs for the given 3D structure.
3. Remove the redundant templates for those with root mean square deviation (RMSD) ≤ 1.5 Å for the same motif, same size, and identical sequence.
4. Collect all the nonredundant motif structures to construct a template library. Table 1 shows the statistics for the current template library.

3 Methods

To predict RNA 3D structures, Vfold first predicts the 2D structures from the sequence. Using the 2D structures as constraint, the model then predicts the corresponding 3D structures.

The key of the free energy-based RNA 2D structure prediction is the enthalpy and entropy parameters used to evaluate the stability of sampled structures. The enthalpy and entropy for the canonical and mismatched base stacks are calculated from Turner’s experimental data. The loop entropies are from the Vfold pre-tabulated parameters.

$$\Delta G_{\text{loop}} = -k_{\text{B}} T \ln Q_{\text{loop}}, \quad Q_{\text{loop}} = \sum_{\text{mismatches}} e^{-(\Delta G_{\text{mm}} - T \Delta S_{\text{loop}})/k_{\text{B}} T}$$

Fig. 2 Ensemble of a 9-nt hairpin loop closed by an A–U base pair, containing five different arrangements of mismatched base stacks within the loop

Here ΔG_{mm} is total free energies of the mismatched base stacks and ΔS_{loop} is the loop entropy for the given intra-loop mismatch constraints.

4. Assign the free energy for each sampled 2D structure:
 $\Delta G_s = \Delta G_{\text{helix}} + \Delta G_{\text{loop}}$.
5. Calculate the total partition function as the sum over all the possible (2D) structures:

$$Q_{\text{tot}} = \sum_{\text{structures}} e^{-\Delta G_s / k_B T}$$

6. Following the similar procedure as above for the total partition function, compute the conditional partition function Q_{ij} for all the 2D structures with nucleotides i and j base paired.
7. Calculate the probability of forming the (i, j) base pair:
 $p_{ij} = Q_{ij} / Q_{\text{tot}}$.
8. From the base pairing probability for all the possible (i, j) pairs, extract the predicted most probable (*see Note 7*) as well as alternative structures.

We use the glycine riboswitch (PDB: 3owi) as an example to show how Vfold predicts the 2D structure. Given the 84-nt RNA sequence of the glycine riboswitch (*see Note 8*), Vfold calculates the base pairing probabilities p_{ij} for all the possible base pairs. The predicted most probable 2D structure (*see Note 7*) can be predicted from p_{ij} , as shown in Fig. 3. It should be noted that depending on the sequence, the Vfold model predicts all the stable structures, including the most probable (most stable) structure as well as the alternative (metastable) structures. Therefore, it is recommended to also find out the possible alternative structures from the base pairing probabilities.

3.2 RNA 3D Structure Prediction for a Given 2D Structure

The Vfold model predicts the 3D structure from a 2D structure by assembling motif-specific structural templates. Currently, due to the limited structural template database, Vfold can only predict the 3D structures with hairpin loops, internal/bulge loops, multi-branched junctions and pseudoknots.

1. Identify the structure motifs (such as hairpin loop, internal loop, pseudoknot loop, and three-way junction) from the given 2D structure.
2. Build the virtual bond 3D structure for helices according to the A-form helix template.
3. For each non-helix motif, search for the best templates from the template library. The search criteria are based on the size (first) and sequence (second) matches (*see Note 9*).
4. From the (all-atom) templates found in the previous step (*see Notes 10 and 11*), build the virtual bond 3D structures of each motifs.

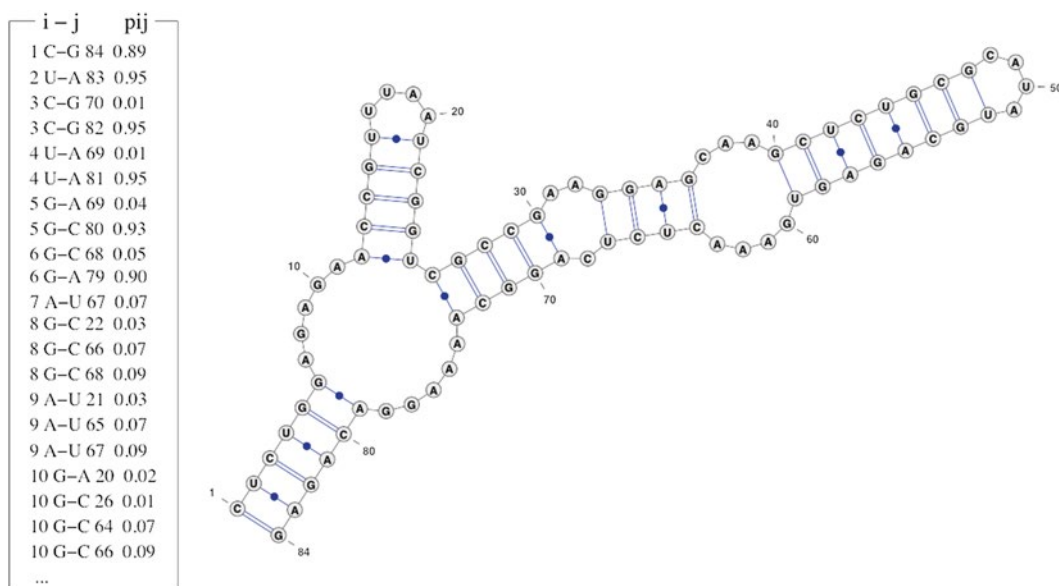


Fig. 3 *Left*: Partial list for the predicted base pairing probabilities for the glycine riboswitch. The first two columns are the position and the nucleotide type of residue i ; the fourth and fifth columns are the nucleotide type and position for residue j ; the last column is the probability of forming the i, j base pair. *Right*: predicted most probable 2D structure derived from the base pairs with probability p_{ij} around 0.90 (e.g., 0.89, 0.95)

5. Assemble the virtual bond 3D structure of the motifs to construct the 3D scaffold of the whole RNA.
6. Add bases to the virtual bond backbone according to the templates for base configurations (*see* **Note 12**).
7. Refine the 3D structure by AMBER energy minimization (*see* **Note 13**).

We use the glycine riboswitch (PDB: 3owi) case for the illustration of the 3D structure prediction. For the purpose of 3D structure prediction, we list only the canonical base pairs in the 2D structure and treat noncanonical base pairs as part of the loop/junction structure (*see* Fig. 3). The predicted 2D structure shows a three-way junction, two internal loops, two hairpin loops and five helices. If the motif structure in the glycine riboswitch is included in the template library, as shown in Fig. 4a, the RMSD between the predicted structure and the experimentally determined structure is 6.3 Å. This RMSD is smaller than the previous prediction of 7.24 Å, shown in Fig. 4b, which excluded the native templates in the template library.

It should be noted that even with the native templates included in the template library, the predicted structure still shows a non-zero RMSD with the PDB structure. The small difference between the A-form helix and the real (slightly distorted) RNA helix (*see* **Note 14**) could result in a notable structural difference in the global fold.

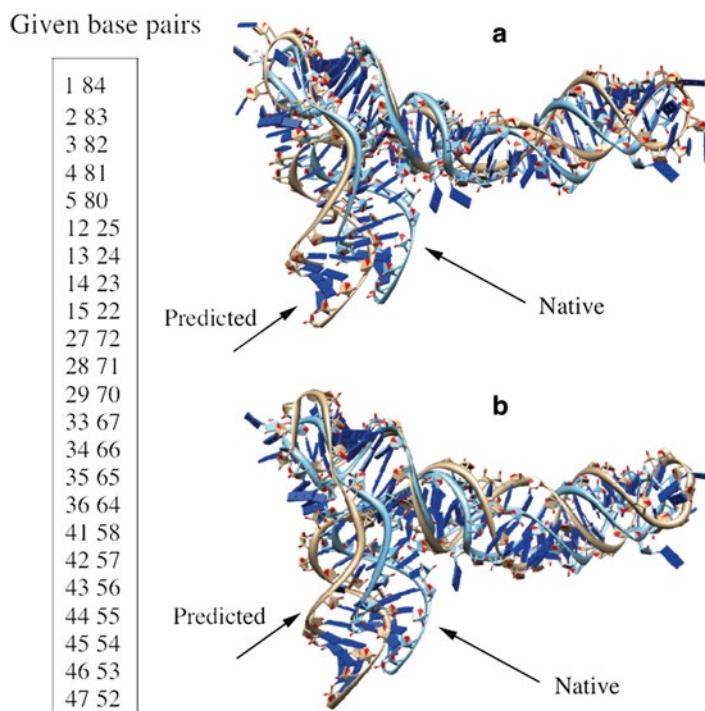


Fig. 4 Given the base pairs shown in the *left*, the predicted 3D structures **(a)** with and **(b)** without including templates from the native structure in the template library, respectively, and the comparisons with the PDB structures (PDB: 3owi) for the glycine riboswitch. The RMSDs are **(a)** 6.3 Å and **(b)** 7.2 Å , respectively

As another example, we predict the 3D structure for the G310-U376 domain of MLV RNA (*see Note 15*). Vfold correctly predicts the 2D structure (*see Note 16*) (*see Fig. 5*). If the native motif structure (PDB: 1s9s) is not included in the template library, we find a larger RMSD between the predicted and the PDB structure.

Because the template-based 3D structure prediction algorithm relies on the knowledge of the known structures, we can realistically expect continuous improvements in the quality of the structure prediction as more and more structures are solved.

4 Notes

1. A survey of the known structures suggests that the virtual bonds (P-C4' and C4'-P) have bond length of ~ 3.9 Å, and have bond angles of (β_c , β_p) in the range of 90–120°.
2. The list of the 2,621 PDB structures used for constructing the template library includes all the PDB entries released before January of 2014. It includes RNA-involved complexes except RNA/DNA hybrids.

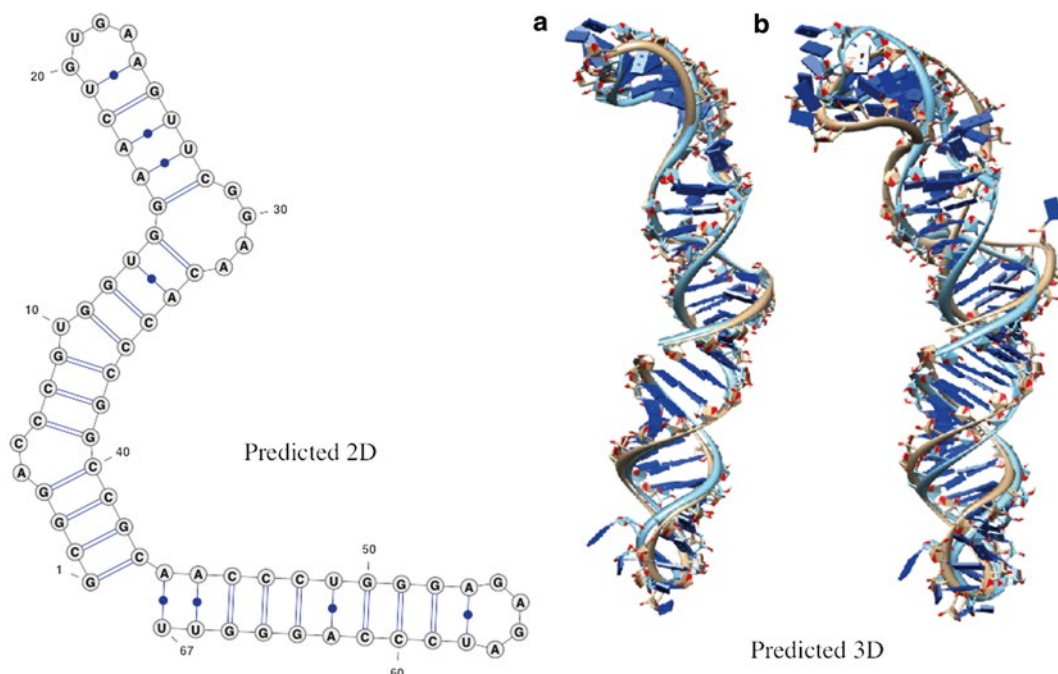


Fig. 5 The prediction of the G310-U376 domain of MLV RNA. The RMSDs between the predictions (a) with and (b) without templates from the native structure and the experimentally determined all-atom structure (PDB: 1s9s) are 3.1 Å and 7.0 Å, respectively. Vfold correctly predicts its 2D structure, shown in the *left*

3. The computational time scales with the chain length N as $O(N^6)$ and the memory scales as $O(N^2)$.
4. The thermodynamic parameters for the different base stacks are from the experimental data (Turner parameters). We use Vfold derived loop entropies to evaluate the loop free energy.
5. The nearest-neighbor model of RNA structure assumes that the stability of a base pair depends only on its adjacent bases, which could be either base-paired (a stacking contribution) or unpaired (a mismatch contribution).
6. A mismatched base stack is formed by a canonical base pair (A-U, G-C, or G-U) and a noncanonical base pair.
Consecutive noncanonical base pairs are considered to be unstable and are not accounted for in the intra-loop mismatch rearrangements.
7. The predicted most probable 2D structure is formed by base pairs of the largest base pairing probabilities.
8. The sequence of the glycine riboswitch is: 5'CUCUGGAGA GAACCGUUAUAUCGGUCGCCGAAGGAG-CAAGCU CUGCGCAUAUGCAGAGUGAAACUCUCAGGCAAAA GGACAGAG3'.

9. To find the best template for a loop, Vfold screens the template library according to the loop size (first criteria) and the sequence (second criteria) matches. If necessary, this may involve sequence replacement in order to match the sequences in the template library. Vfold defines the sequence distance $H = \sum_i h^i$ to find the optimal templates. Here, h^i is the hamming distance between nucleotide i in the selected template and the corresponding nucleotide in the target sequence through the following substitution cycles: $A \rightarrow G \rightarrow C \rightarrow U$, $C \rightarrow U \rightarrow A \rightarrow G$, $G \rightarrow A \rightarrow U \rightarrow C$, $U \rightarrow C \rightarrow G \rightarrow A$.
10. If there is no templates available for a motif, no 3D structures will be predicted.
11. There might have more than one optimal templates available for the same motif. This can lead to more than one predicted 3D structures.
12. For the nucleotides in helices, the base atoms are added following the A-form helix.
13. The all-atom energy (such as AMBER) minimization causes only small change in the RMSD of the structure.
14. Helices contain canonical base pairs only: A-U, G-C, and G-U base pairs. The RMSD between a helix of known RNA structures and the standard A-form helix is $<1.2 \text{ \AA}$.
15. The sequence of the G310-U376 domain of MLV RNA is:
5' G C G G A C C C G U G G U G G A A C U G U G
AAGU-UCGGAACACCCGGCCGCAACCCUGGGA
GAGAUCCCAGGGUU3'.
16. The base pairs used to predict the 3D structures of MLV RNA:
(1 43), (2 42), (3 41), (4 40), (7 39), (8 38), (9 37), (11 36),
(12 35), (13 34), (14 33), (15 28), (16 27), (17 26), (18 25),
(19 24), (44 67), (45 66), (46 65), (47 64), (48 63), (49 62),
(50 61), (51 60), (52 59), (53 58).

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Post-crystallization Improvement of RNA Crystal Diffraction Quality

Jinwei Zhang and Adrian R. Ferré-D'Amaré

Abstract

The crystallization and structural determination of large RNAs and their complexes remain major bottlenecks in the mechanistic analysis of cellular and viral RNAs. Here, we describe a protocol that combines post-crystallization dehydration and ion replacement that dramatically improved the diffraction quality of crystals of a large gene-regulatory tRNA–mRNA complex. Through this method, the resolution limit of X-ray data extended from 8.5 to 3.2 Å, enabling structure determination. Although this protocol was developed for a particular RNA complex, the general importance of solvent and counterions in nucleic acid structure may render it generally useful for crystallographic analysis of other RNAs.

Key words X-ray crystallography, Crystal dehydration, Ion replacement, Riboswitch, T-box RNA, tRNA

1 Introduction

The rapid exploration of the noncoding genome using high-throughput technologies is revealing critical roles for RNA structure in a wide range of cellular processes [1]. In addition, many DNA and RNA viruses utilize defined three-dimensional RNA folds to enable their lifecycle and achieve infectivity [2–4]. Despite critical roles of structured RNAs in biology and their impact on human health, their functional elucidation is hampered by a paucity of available structural information [4, 5]. One hundred years after its invention, X-ray crystallography still provides unparalleled structural information for macromolecules. The rarity of diffraction-quality crystals of larger RNAs (longer than 100 nucleotides) remains a major roadblock that hinders their structure determination [4]. Compared to proteins, the polyanionic nature of the RNA backbone, reduced chemical diversity of the four nucleobases, reduced presence of long-range contacts, as well as inherent conformational flexibility, render it difficult for RNAs to form specific, stable crystal packing contacts [5].

Many RNA crystals only diffract X-rays to resolutions in the range of 5–8 Å, insufficient to provide biochemical insight (~3.5 Å or better is desirable). For protein crystals, many post-crystallization treatment strategies such as annealing, dehydration, supplementing ligands, etc., have been developed [6–9]. For RNA crystals, the favorable response of crystals of *glmS* riboswitch-ribozyme to dehydration hints that the diffraction quality of RNA crystals could also be significantly improved by post-crystallization treatments [10, 11]. In order to facilitate the development of general strategies and methods for crystallographic studies of larger RNAs, we detail and rationalize a protocol that enabled the crystallization and structure determination of a large tRNA–mRNA complex [12, 13]. Exploiting the general importance of RNA solvation and counterions in stabilizing compactly folded RNAs [14], this method concurrently dehydrates the RNA crystals and substitutes the divalent cations in them. This two-pronged approach drives rigid-body movements of the RNA complexes in the crystal, causing them to achieve geometrically and energetically superior packing.

2 Materials

1. Oligonucleotides for PCR amplification.
2. *Taq* DNA polymerase, 5,000 U/mL.
3. T7 RNA polymerase, 50,000 U/mL.
4. Diethylpyrocarbonate (DEPC)-treated water (*see Note 1*).
5. RNA Binding Buffer: 50 mM HEPES–KOH, pH 7.0, 100 mM KCl, 20 mM MgCl₂, 5 mM tris (2-carboxyethyl) phosphine (TCEP).
6. 20 mM spermine solution, in DEPC-treated water, filtered through 0.2 µm filter.
7. Crystallization Solution: 50 mM Bis-Tris (HCl) pH 6.5, 0.3 M Li₂SO₄, 20 mM MgCl₂, 20 % (w/v) polyethylene glycol (PEG) 3350.
8. EasyXtal 15-Well Tool (Qiagen).
9. MicroSieves and MicroSaws (MiTeGen).
10. 90° angled MicroLoops or MicroMounts (MiTeGen).
11. Crystal Treatment Solutions: 50 mM Bis-Tris (HCl), pH 6.5, 100 mM KCl, 20–50 mM SrCl₂ or 20–100 mM MgCl₂, 40–45 % PEG3350, 5 mM TCEP.

3 Methods

3.1 Design and Synthesis of T-Box RNA and tRNA for Crystallization

1. Initial biochemical and biophysical characterization of T-box RNA-tRNA complexes [15] was essential for design and engineering of crystallization constructs (Fig. 1). Glycine-specific *glyQ/glyQS* T-box constructs from 20 species were selected from a multiple sequence alignment, with preference given to thermophilic, extremophilic, and pathogenic organisms. The T-box and tRNA constructs were transcribed in vitro using

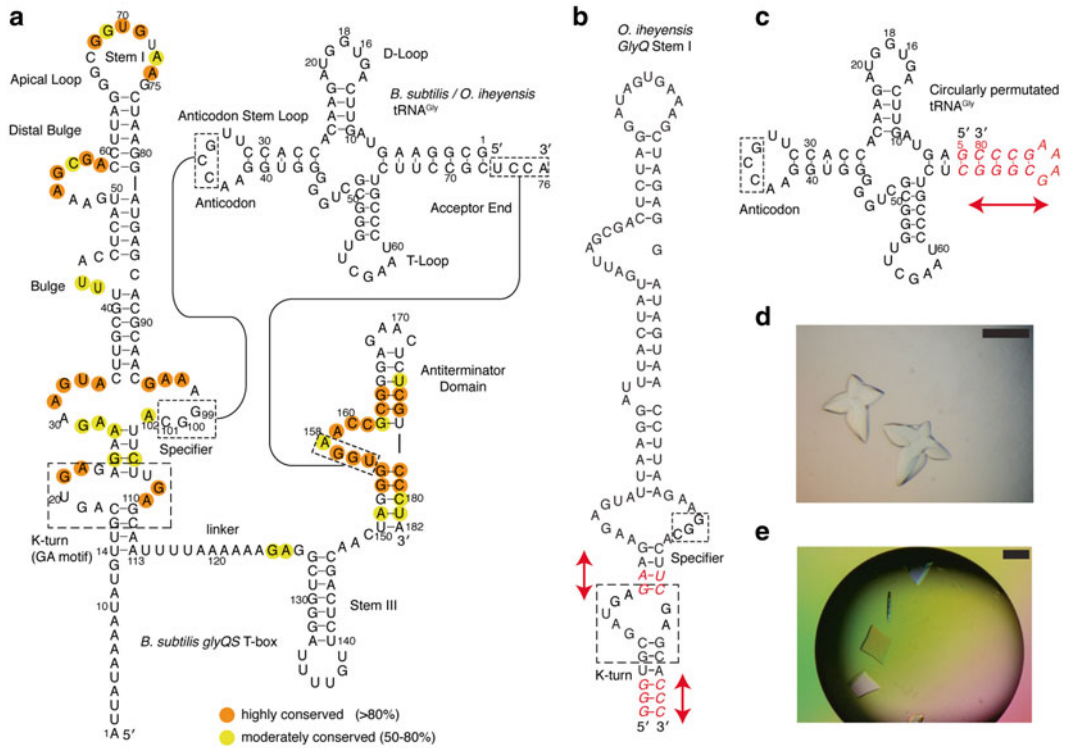


Fig. 1 Sequences and secondary structures of full-length and truncated T-box riboswitch RNA used for crystallization. **(a)** Secondary structure and sequence conservation of a full-length *B. subtilis* glycine-responsive *glyQS* T-box riboswitch and its cognate tRNA^{Gly}. Circles with *dark* and *light orange* shades indicate highly conserved (>80 %) and moderately conserved (50–80 %) sequences, respectively. Salient structural features on both RNAs are *boxed* and *annotated*. Intermolecular T-box-tRNA base-pairing interactions are indicated by *solid lines* connecting the boxed sequences. **(b)** Secondary structure of *Oceanobacillus iheyensis* *glyQ* T-box Stem I domain used for co-crystallization. The *italic, red* sequences and *red arrows* denote engineered regions. **(c)** Secondary structure of engineered *B. subtilis*/*O. iheyensis* tRNA^{Gly} (identical sequences) used for co-crystallization. The original tRNA acceptor stem sequence is circularly permuted and capped with a stable GAAA tetraloop (*red, italic* sequences). The *bidirectional arrow* denotes the length variations to screen for optimal crystal contacts. **(d)** Representative crystals of T-box-Stem I-tRNA complexed with *Methanococcus jannaschii* L7Ae. All scale bars represent 200 μm. **(e)** Representative crystals of T-box-Stem I-tRNA complexed with *B. subtilis* YbxF. Note the differences in crystal morphology as dictated by the protein component in the complex

T7 RNA Polymerase, purified by denaturing Urea-PAGE, electroeluted, washed once with 1 M KCl and extensively with DEPC-treated water, concentrated, and stored at 4 or -20°C before use [11, 16].

2. T-box RNAs from a range of bacterial species were evaluated for their propensity to form monodisperse, stoichiometric complexes with tRNA using non-denaturing PAGE. T-boxes from a handful of bacterial species, such as the extremely halotolerant and alkaliphilic *Oceanobacillus ibeyensis* eventually used in structural determination, exhibited robust tRNA binding, forming tRNA–mRNA complexes that migrated as relatively sharp bands on non-denaturing gels.
3. Full-length T-box RNAs that contain both the Stem I and the antiterminator domains (Fig. 1a) exhibited a tendency to form dimers, presumably due to the thermodynamic instability of the antiterminator [17]. Therefore, T-box RNAs were truncated at a series of lengths and their affinities towards tRNA and tendency to form monodisperse complexes evaluated using isothermal titration calorimetry (ITC) and non-denaturing gels. This analysis demonstrated that Stem I is the minimal T-box domain that is both necessary and sufficient for high-affinity, specific binding to tRNA (Fig. 1b–c) [12].
4. To aid crystallization of RNA, several RNA-binding proteins have been successfully used as crystallization chaperones, such as the human spliceosomal U1A protein [18] and recombinant antibody fragments (Fabs) [19, 20]. The Kink-turn (K-turn) is a widespread bistable RNA structural motif initially discovered on the ribosome that sharply kinks the RNA duplex backbone by 120° and is the landing platform to recruit several conserved proteins to accomplish a range of cellular functions [21–24]. The T-box Stem I domain harbors a conserved, functionally important K-turn [22]. To stabilize the bistable K-turn structure, provide added opportunities for crystal packing, and allow for phasing using selenomethionines, a panel of K-turn binding proteins is tested for their ability to support crystal growth and improve crystalline order. Interestingly, the choice of K-turn binding protein appreciably influenced the crystal morphology. While the presence of thermophilic *Methanococcus jannaschii* L7Ae protein [25] (and other species of L7Ae) yielded star-shaped non-single crystals (Fig. 1d), the addition of mesophilic *B. subtilis* YbxF [26] produced single, square-plate-shaped crystals (Fig. 1e). The latter is much more amenable to diffraction data collection. In the absence of any K-turn-binding protein, only non-diffracting crystals of T-box–tRNA binary complexes were occasionally observed.

3.2 Crystallization of the T-Box Stem I-tRNA-YbxF Ternary Complex

1. Dilute concentrated tRNA^{GAAA} (~1 mM; 24 g/L) to ~20 μ M using DEPC-treated water to reduce intermolecular interaction and dimerization.
2. “Snap-cool” tRNA^{GAAA} by incubating at 90 °C for 3 min followed by rapid cooling to 4 °C using a thermocycler (*see Note 2*).
3. Concentrate refolded tRNA to ~12 g/L (500 μ M) using Amicon spin concentrators (10 kDa MWCO; 0.5 mL).
4. Mix 200 μ M each T-box Stem I RNA and snap-cooled tRNA in RNA Binding Buffer (Materials), incubate first at 50 °C for 10 min and then at 37 °C for 30 min.
5. Add one equivalent selenomethionyl *B. subtilis* YbxF to the RNA complex.
6. Add spermine to 2 mM. The mixture may become transiently cloudy. Mix gently with a pipette tip.
7. Heat to melt a stock of 2 % low-melting-point agarose solution and allow it to cool to 37 °C using a heat block to prevent it from solidifying.
8. Mix 1:1 the sample solution and Crystallization Solution, keep at 37 °C.
9. Add 1/10 volumes of 2 % low-melting-point agarose solution and gently mix by pipetting up and down. The presence of agarose fibers in crystal solvent channels has been shown to lend mechanical support to the crystals [27, 28]. The presence of 0.2 % low-melting-point effectively prevents the T-box co-crystals from cracking induced by the sudden change in osmolarity (Fig. 2a).
10. Transfer the crystallization mixture onto cover slides and initiate crystallization experiments by hanging drop vapor diffusion.

3.3 Post-crystallization Treatments

1. Square-plate-shaped crystals of the T-box-tRNA-YbxF ternary complex start appearing as early as 1–2 days. Diffraction quality crystals tend to grow more slowly, reaching final dimensions of $300 \times 300 \times 50 \mu\text{m}^3$ over the course of 1–3 weeks (Fig. 2a). These crystals have the symmetry of space group C222₁, with unit cell dimensions of $a=108.7 \text{ \AA}$, $b=108.8 \text{ \AA}$, $c=291.8 \text{ \AA}$. As do many other macromolecular crystals with relatively long unit cell edges, the longest unit cell edge (291.8 $\text{\AA}$) of these crystals is parallel to the shortest physical dimension of the crystals, i.e., the edge that describes the thickness of the rectangular or rhombic plates. Thus, oscillation diffraction images that result from incident X-rays that traverse through the broad faces of the plates suffer from significant overlap of neighboring reflections. Such overlap is circumvented by the use of 90° bent crystal loops, which restrict the incident X-rays to only enter and exit the crystals through their shortest physical “edges” but not their “faces” (Fig. 2a).

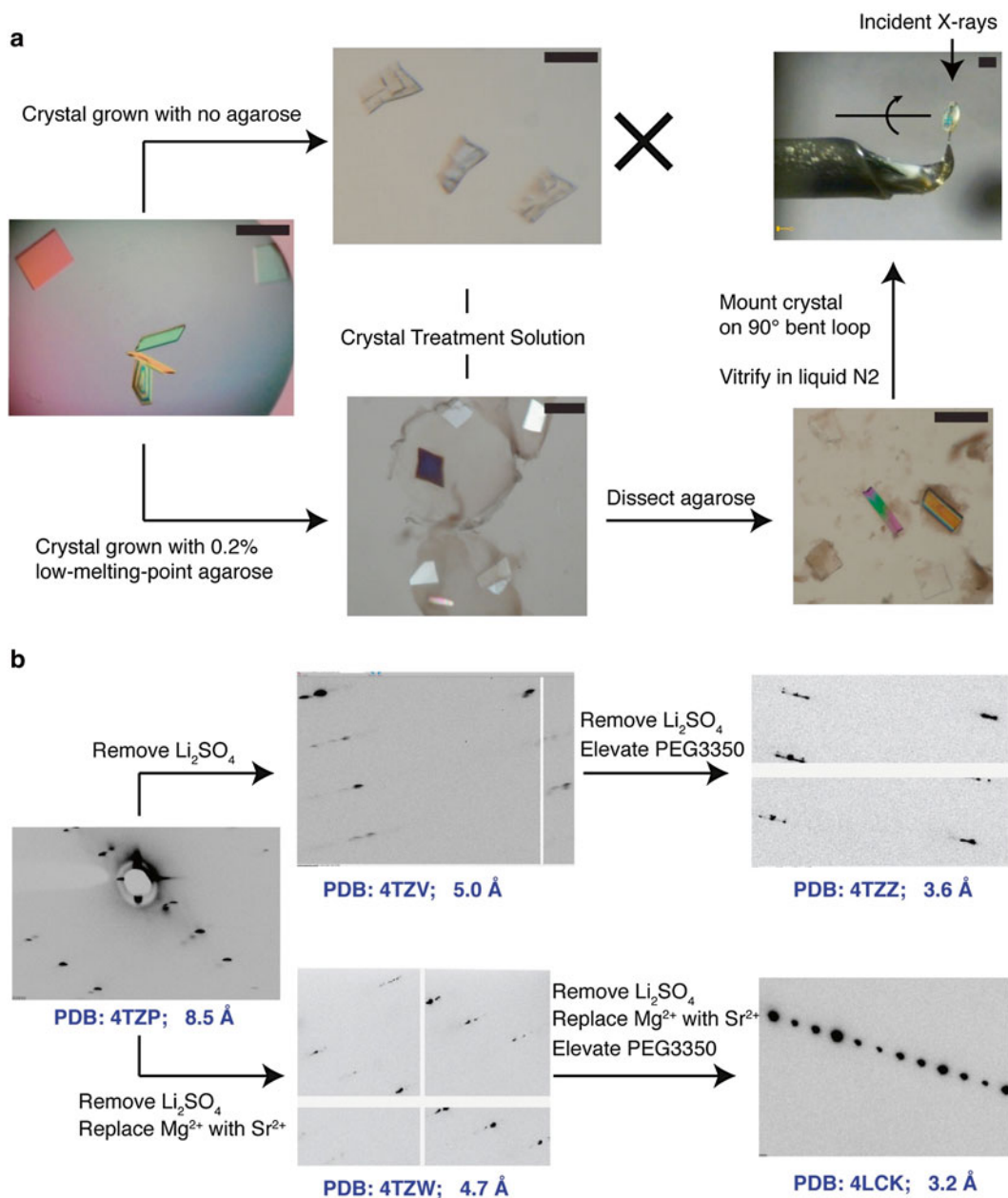


Fig. 2 Post-crystallization treatments dramatically improve diffraction quality of large RNA complexes. **(a)** Post-crystallization treatment procedures and effects on the crystal appearance. Due to the drastic changes in osmolarity (e.g., induced by a 20–40 % change in PEG3350 concentration), pervasive crystal cracking and even disintegration occurs. Cracking is effectively prevented by the mechanical support from the agarose fibers in the solvent channels of the crystals. Note the agarose network that transferred together with the embedded crystals. All scale bars denote 200 μ m. **(b)** Comparison of magnified portions of diffraction oscillation photographs of untreated (as-grown) crystals (*left*, PDB: 4TZP), partially treated crystals (*middle panels* and *top right panel*, PDB: 4TZV, 4TZW, and 4TZZ), and crystals that were subjected to full cation replacement and dehydration (*lower right panel*; PDB: 4LCK) to demonstrate the improvement in spot profile and order-to-order separation. *Arrows* indicate progressive additions of treatments. Diffraction limits are indicated below each panel. Post-crystallization treatment and resulting crystal properties are summarized in Table 1

2. Depending on the final concentration of low-melting point agarose in the crystallization drop and temperature, the entire drop may exhibit consistencies ranging from fluid liquid to viscous liquid, to jelly-like solid to robustly solid. Select appropriate tools to transfer crystals into ~200 μ L Crystal Treatment Solutions in glass depression plates, i.e., use conventional nylon loops to transfer individual crystals from non-viscous liquid drops, and use tools such as MicroSieves (MiTeGen) to transfer whole, solidified drops. The composition of Crystal Treatment Solutions will vary as it is based on both the RNA Binding Solution and the Crystallization Solution. A gradient of concentrations of the primary precipitant (20–50 % PEG3350 in this example) is scouted to achieve a range of final solvent contents and the effect on diffraction quality is measured. Different concentrations of a panel of divalent cations in particular the alkaline earth metals (Mg^{2+} , Ca^{2+} , Sr^{2+} , Ba^{2+}) should be screened, both for supporting crystal growth and for post-crystallization treatment. In the case of the T-box complex crystals, crystal growth in Mg^{2+} combined with post-crystallization treatment in Sr^{2+} stands out as the optimal procedure, producing the best Bragg spots profiles required for *de novo* phasing using single-wavelength anomalous dispersion (SAD).
3. Seal each well of the depression plate using a glass cover slide and Vaseline. Incubate the crystals in Crystal Treatment Solution (Materials) for 16 h. For the crystals of the T-box ternary complex, shorter treatments (i.e., less than 4 h) generally do not produce the full effect of the treatment.
4. Carefully dissect the crystals out from their surrounding agarose network using MicroSaws (MiTeGen) and remove as much as agarose as possible (Fig. 2a). As the orientation of the crystals in the crystal loop is critical for reducing overlap during data collection, it is essential to trim nearly all agarose away from the crystal faces so that the plate-like crystals would be mounted parallel to the plane of the 90° bent loop due to surface tension.
5. Using a 90° bent loop such as the angled MicroLoops or MicroMounts (MiTeGen), pick up single, trimmed crystals and immediately plunge into liquid nitrogen for vitrification. As the Crystal Treatment Solution already contains at least 40 % (w/v) polyethylene glycol (PEG) 3350, no additional cryoprotective agent is necessary.

3.4 Understanding the Basis of Treatment-Induced Improvement of Crystal Quality

1. Structure determination of as-grown, untreated crystals, and a number of crystals subjected to various combinations of post-crystallization treatments (Fig. 2b and Table 1) allow the tracking of macromolecular movements in these crystals in response to the treatments received [12, 13].

2. Structural alignment of untreated and optimally treated crystals reveal that the ternary complexes of T-box-tRNA-YbxF shift closer to each other in the crystal as rigid bodies (Fig. 3a; see Note 3), producing superior packing contacts such as three intimate base-stacking interactions between symmetry-related complexes (Fig. 3b) as well as a stable A-minor interaction between the engineered GAAA tetraloop on tRNA acceptor stem and the minor groove of the proximal region of T-box Stem I (Fig. 3c).

Table 1

Select properties of crystals treated with varying degrees of ion replacement and dehydration

PDB code	Li ₂ SO ₄ (mM)	MgCl ₂ (mM)	SrCl ₂ (mM)	PEG 3350 (% w/v)	Resolution (Å)	Space group	Unit cell dimensions (Å)	V _M (Å ³ /Da)	V _s (%)
4TZP	300	20	0	20	8.5	C222 ₁	108.7, 108.8, 291.8 ^a	3.26	74.6
4TZV	0	20	0	20	5.0	P4 ₃ 2 ₁ 2	75.7, 75.7, 270.2 ^a	2.93	71.7
4TZW	0	0	50	20	4.7	P4 ₃ 2 ₁ 2	75.3, 75.3, 268.9 ^a	2.89	71.3
4TZZ	0	100	0	48	3.6	P2 ₁	70.6, 260.7, 70.7 ^b	2.46	66.3
4LCK	0	0	40	40	3.2	C222 ₁	100.8, 109.7, 268.1 ^a	2.81	70.4

^aV_M Matthews coefficient (Matthews 1968)^bV_s calculated solvent content^aα = β = γ = 90°^bα = γ = 90°, β = 92.8°

Fig. 3 (continued) The rear face of the interdigitated T-loops of Stem I distal region (opposite the face interacting with the tRNA elbow) [12] form a prominent flat surface available for crystal packing [12]. Two patches of this flat surface (A39 and A60 respectively), upon full treatment, engage in direct stacking contact with the apical adenine of the GAAA tetraloop capping the tRNA acceptor stem (tA73) of a second complex (*green*), and with the terminal base pair of T-box Stem I (G1·C102) of a third complex (*teal*), respectively. *Dotted triangle* surrounds an intermolecular A-minor interaction (detail in **c**) present only in the fully treated crystals. tRNA residue numbers are preceded by 't'. **(c)** Detail of the intermolecular class-IA-minor interaction formed between the tetraloop of a tRNA and the minor groove of the proximal region of Stem I, colored as in **(b)**. **(d)** Interfacial Sr²⁺ ions bridge symmetry-related T-box RNA molecules by binding to their 3' termini. A well-defined Sr²⁺ ion (*green sphere*) is seen bound to the *cis*-diol of the T-box RNA 3' terminal nucleotide (C102, *marine*), and two non-bridging oxygen atoms of a symmetry-related T-box molecule (*cyan*), through inner-sphere coordination. Similar inner-sphere coordination between Sr²⁺ and two 3' *cis*-diol groups bridge two symmetry-related heptanucleotide derived from tRNA^{Ala} acceptor stem [30]. The Sr²⁺ bridging two symmetry-related T-box RNA thus may have contributed significantly to the improved crystal quality through Sr²⁺ soaking. **(e)** Pervasive Sr²⁺ binding to RNA nucleobases and backbone, such as a pair of well-defined Sr²⁺ ions next to T-box G43, one of which makes bidentate inner-sphere interactions with the Hoogsteen face of G43. The electron density shown in **(d)** and **(e)** is a portion of a composite simulated anneal-omit 2F_o−|F_c| synthesis contoured at 1.5 s.d. overlaid with the final refined model

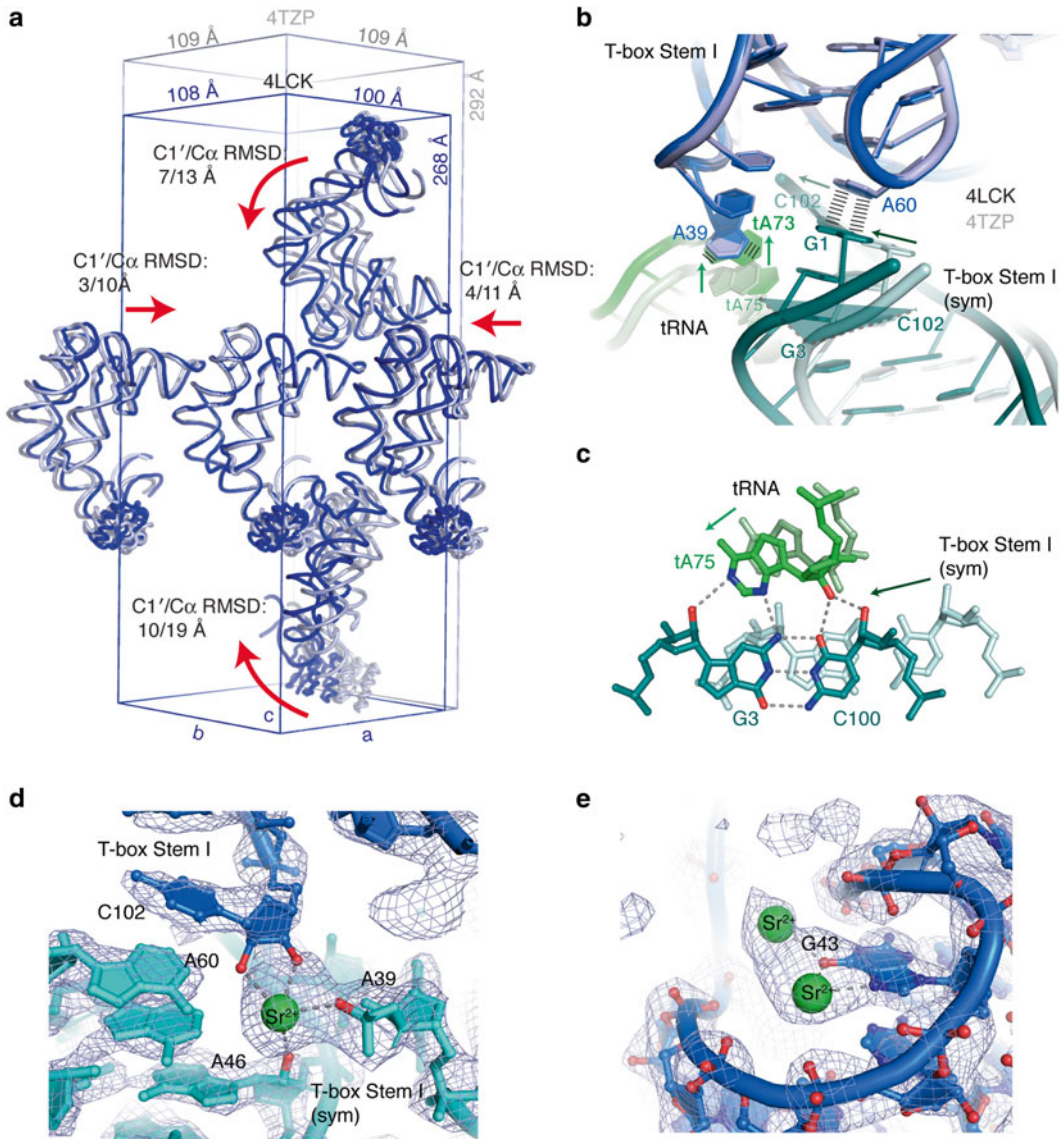


Fig. 3 Treatment-induced, in-crystal movements of T-box ternary complexes produce superior crystal contacts. **(a)** In-crystal redistribution of T-box ternary complexes as rigid bodies driven by dehydration and cation replacement. Overlay of T-box ternary complexes in untreated (as-grown) crystals (*light blue*, PDB: 4TZP) and fully dehydrated and cation-exchanged crystals (*dark blue*, PDB: 4LCK). The corresponding crystallographic unit cells are also shown, indicating close to ~10 % compression along both *a* and *c* axes. The reference complexes in the center of the panel superimpose well (RMSD for 172 C1' < 1.4 Å), but the neighboring four complexes shift substantially closer as a result of the post-crystallization treatment (RMSDs range from 3 to 10 Å and 10 to 19 Å, for RNA C1' and protein Cα, respectively). *Red arrows* denote directions of displacement (translation and rotation) of the four neighboring complexes. **(b)** Treatment-induced formation of an intimate crystal contact involving three symmetry-related T-box ternary complexes, shown in *blue*, *green*, and *teal*, respectively. Molecules from the untreated (PDB: 4TZP) and fully cation-replaced and dehydrated crystals (PDB: 4LCK) are overlaid and colored in *pastel* and *solid colors*, respectively. *Parallel lines* denote intermolecular stacking between nucleobases of symmetry-related complexes. *Arrows* indicate displacements between the untreated and fully treated states.

3. The unique preference for Sr^{2+} in post-crystallization treatments of T-box co-crystals may be rationalized by its specific association with the 3' *cis*-diols of neighboring symmetry-related T-box RNAs (Fig. 3d), its frequent bidentate innersphere interactions with the Hoogsteen faces of purines (Fig. 3e), or its presence at bulges and junctions where phosphates cluster, or bridging across the narrow major groove. The ability of Sr^{2+} to bind RNA 3' termini and its flexible coordination geometry are properties that may allow it to improve crystalline packing of RNA [29].

4 Notes

1. DEPC is a toxic alkylating agent. It should be handled with appropriate personal protective equipment in a chemical fume hood. DEPC-treated water is nontoxic, because after mixing, the water-DEPC mixture is autoclaved. Heating in the presence of water converts DEPC into nontoxic carbon dioxide and ethanol.
2. tRNA^{Gly} and other tRNAs are known to form dimers in solution depending on conditions used for folding the RNA. To reduce dimerization, tRNAs are diluted in DEPC-treated water and “snap-cooled,” which favors tRNA folding while suppressing intermolecular association.
3. Note that the space group and the unit cell dimensions of the crystals have changed significantly in response to the post-crystallization treatments (Table 1).

Acknowledgements

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Chapter 3

Expression and Purification of RNA–Protein Complexes in *Escherichia coli*

Margueritte El Khouri, Marjorie Catala, Bili Seijo, Johana Chabal, Carine Tisné, Frédéric Dardel, and Luc Ponchon

Abstract

For structural, biochemical or pharmacological studies, it is required to have pure RNA in large quantities. We previously devised a generic approach that allows efficient *in vivo* expression of recombinant RNA in *Escherichia coli*. We have extended the “tRNA scaffold” method to RNA/protein co-expression in order to express and purify RNA by affinity in native condition. As a proof-of-concept, we present the expression and the purification of the AtRNA-mala in complex with the MS2 coat protein.

Key words Expression plasmid, Recombinant RNA, RNA purification, tRNA scaffold, Protein expression

1 Introduction

Recombinant transfer RNA (tRNA) has been successfully expressed *in vivo* in *Escherichia coli* [1–4]. This is possible because tRNAs are recognized and processed by a number of cellular enzymes. Furthermore, as a consequence of their three-dimensional structure, tRNAs are extremely stable to both heat-unfolding and nucleases [5]. This allows them to escape degradation and accumulate in *E. coli* when overexpressed from a recombinant plasmid. We took advantage of these specific features to express recombinant RNA as chimeras, by inserting them into the protective scaffold of a tRNA [6, 7].

We developed a new strategy to perform RNA–protein co-expression using *E. coli*. This approach is derived from the armored RNA technology [8]. The Armored tRNA (AtRNA) is composed of three parts: a tRNA scaffold, the MS2 operator hairpin and the RNA cloning site (Fig. 1a). Concretely, we designed two plasmids for the His6-MS2 coat protein expression and the AtRNA expression, respectively the pACYCT2-coat and the pBSKrna-AtRNA. The pACYCT2 and the pBSKrna are compatible with two different

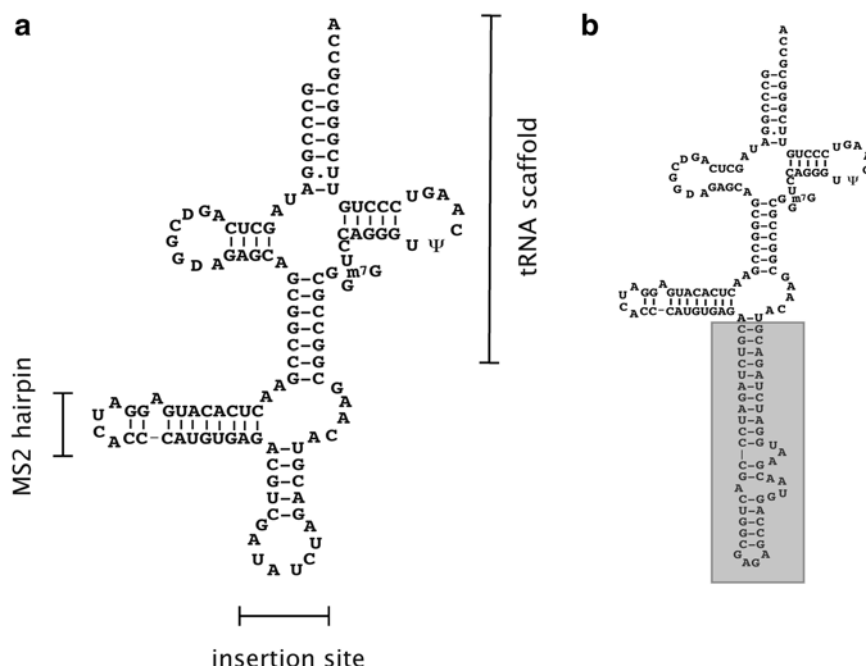


Fig. 1 (a) Schematic structure of standard AtRNA. The AtRNA is composed of three parts: tRNA scaffold, the MS2 operator hairpin, and the RNA cloning site. The insertion site was designed to maintain the hairpin structure. (b) Secondary structure of the AtRNA-mala. The malachite green aptamer (boxed) is inserted into the AtRNA scaffold

origin of replication and they contain, respectively, the ampicillin and chloramphenicol resistance genes, allowing selection of bacteria only co-transformed by the two plasmids. The induction of the protein expression is under the control of the *lac* operator and is thus triggered by the addition of IPTG. The His6-MS2 coat protein and the AtRNA are coproduced and copurified in one step by affinity on NiNTA. Here, As a proof-of-concept, we described the purification of the AtRNAmala, an AtRNA which the malachite green aptamer is fused to the AtRNA (Fig. 1b).

The malachite green aptamer remains functional under these production conditions. Our system allows the production the purification of any RNA under native conditions without denaturation steps [9].

2 Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water to attain a sensitivity of 15 MΩ cm at 25 °C) and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise).

2.1 RNA/Protein Expression

1. RNA expression vector pBSK_{rna}-AtRNA_{mala} derived from the pBSTNAV plasmid (*see Note 1*).
2. Protein expression vector pACYCT2-coat derives from the pACYCduet plasmid (Novagen) (*see Note 1*).
3. Electrocompetent XL1-Blue *E. coli* cells (*see Note 2*).
4. 1 M Isopropyl β -D-1-thiogalactopyranoside (IPTG). Filter-sterilize and store at -20°C .
5. 2 $\times$ TY medium: Add 16 g tryptone, 10 g yeast extract, and 5 g sodium chloride to 1 L H₂O and sterilize by autoclaving.
6. 100 mg/mL ampicillin. Filter-sterilize and store at -20°C .
7. 20 mg/mL chloramphenicol. Filter-sterilize and store at -20°C .
8. Luria Broth (LB).
9. LB agar plates Add 12 g Bacto agar to 1 L LB medium before autoclaving. To prepare plates, allow medium to cool until flask or bottle can be held in hands without burning, then add 1 mL appropriate ampicillin stock(s), mix by gentle swirling and pour or pipette approximately 30 mL into each sterile petri dish (100 mm diameter). The final concentration of ampicillin should be 100 $\mu\text{g/mL}$ and of chloramphenicol should be 20 $\mu\text{g/mL}$.
10. Electroporator and electroporation cuvettes.
11. Temperature-controlled shaking incubator.
12. 1-L culture flasks.
13. Apparatus for gel electrophoresis of RNA and protein.
14. Loading Buffer: 50 % (v/v) glycerol, 0.2 % (w/v) xylene cyanol, 1 % SDS and 10 mM Tris-HCl pH 8.6.
15. Handheld UV lamp.
16. Shrink wrap.
17. Coomassie staining solution: 0.2 % Coomassie Brilliant Blue R250, 45 % ethanol, 10 % acetic acid.
18. Coomassie destaining solution: 20 % ethanol, 10 % acetic acid, 1 % glycerol.
19. Water-saturated phenol.
20. RNA extraction buffer: 10 mM MgAcetate, 10 mM MgCl₂, pH 7.4.

2.2 RNA/Protein Purification

1. Mechanical device to disrupt *E. coli* cells (e.g., a sonicator, French press or cell homogenizer).
2. Buffer A: 20 mM Tris-HCl and 300 mM NaCl, pH 8.
3. Buffer B: 20 mM Tris-HCl and 300 mM NaCl, 1 M Imidazole, pH 8.
4. AKTA FPLC chromatography system (or equivalent).
5. 5 mL Ni-NTA resin.

3 Methods

3.1 Cell Growth

1. Co-transform 50 μL of electrocompetent XL1-Blue with 10–100 ng of Chimeric RNA expression vector and of His6-MS2 coat protein fusion and spread 50 μL on an agar plate containing 100 $\mu\text{g}/\text{mL}$ ampicillin and 20 $\mu\text{g}/\text{mL}$ chloramphenicol. Incubate the plate overnight at 37 °C.
2. Inoculate 100 mL 2xTY medium containing 100 $\mu\text{g}/\text{mL}$ ampicillin and 20 $\mu\text{g}/\text{mL}$ chloramphenicol in a 500-mL baffle-bottomed shake flask with a colony from the transformation; Shake overnight at 220 r.p.m. and 37 °C.
3. Add 25 mL of the saturated overnight culture to 1 L fresh 2xTY medium containing 100 $\mu\text{g}/\text{mL}$ ampicillin and 20 $\mu\text{g}/\text{mL}$ chloramphenicol in a baffle-bottomed shake flask.
4. Shake the flasks at 250 r.p.m. and 37 °C until the cells reach mid-log phase $\text{OD}_{600 \text{ nm}}$ approximately 0.6.
5. Collect a sample for protein production analysis (*see Note 3*).
6. Add IPTG to a final concentration of 1 mM. Continue shaking for 3–4 h.
7. Collect a second sample for protein production analysis (*see Note 3*).
8. Pellet 5 mL of culture by centrifugation for 10 min at $4,000 \times g$ and 4 °C for RNA production analysis.
9. Resuspend the cell pellet in 180 μL of RNA extraction Buffer. Add 200 μL of water-saturated phenol (*see Note 4*). Agitate gently for 20 min at room temperature in a polypropylene conical tube. Centrifuge for 10 min at $10,000 \times g$ and collect the 150 μL of aqueous phase.
10. Add 0.1 volume of 5 M NaCl and 2 volumes of ethanol. Centrifuge 10 min at $10,000 \times g$ at 4 °C in a polypropylene conical tube. Dissolve the pellet in 30 μL of water. Add 10 μL of loading buffer. Preserve for electrophoresis analysis.
11. Recover the cells by centrifugation for 15 min at $6,000 \times g$ and 4 °C. Freeze the cell pellet at –80 °C until further use (this may be for as long as several months).
12. Analyze the fractions by electrophoresis on 11 % SDS-PAGE. Following electrophoresis, stain the gel with staining solution and destain with destaining solution. Place the gel on shrink wrap (*see Note 5*) and place on a white paper. Reveal the RNA by UV shadowing with the handheld UV lamp placed above the gel. The protein appears as a blue band and RNA and grey band (Fig. 2).

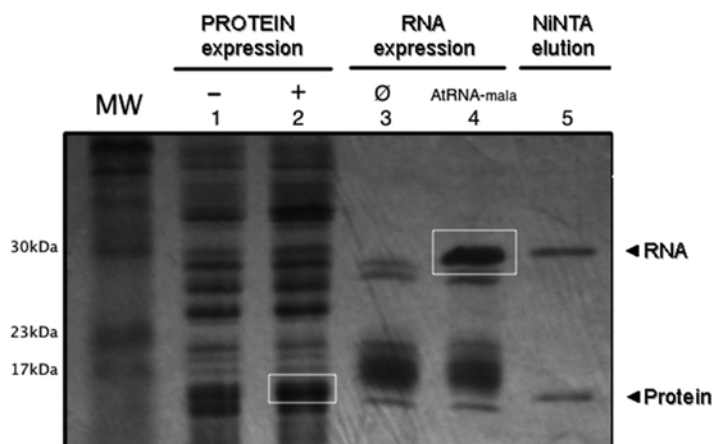


Fig. 2 Co-expression of AtRNA-mala/His6-MS2 coat protein in XL1-Blue *E. coli*. Crude bacteria extracts (before and after IPTG induction –/+) and crude RNA minipreps were separated on a 16 % PAGE-SDS gel and visualized by Coomassie staining and UV shadowing. Ø (control): bacteria transformed by the vector with no insert. *White boxes* indicate the overexpressed RNA and protein. NiNTA elution: RNA and protein are eluted in the same fractions. The molecular weight (MW) of protein standards is given in kDa on the *left*. The *black arrows* indicates the AtRNA-mala and the protein

3.2 RNA–Protein Complex Purification

Perform all of the following procedures at 4 °C.

1. Resuspend the cell pellet in Buffer A, using at least 10 mL/g wet cell paste.
2. Lyse the cell suspension using mechanical device to disrupt *E. coli* cells (e.g., a sonicator, French press or cell homogenizer) and centrifuge the disrupted cell suspension for at least 30 min at 15,000 × *g*.
3. Load the supernatant onto a column of Ni-NTA resin equilibrated with Buffer A (*see Note 6*).
4. Wash the column with buffer A; 20 mM imidazole until a stable baseline is reached and then elute the RNA–protein complex with a linear gradient over 10 column volumes into buffer B.
5. Analyze the fractions by electrophoresis on 11 % SDS-PAGE as described in the previous paragraph (Fig. 2) (*see Note 7*).

4 Notes

1. The aptamer for the malachite was cloned between the EagI and SacII restriction sites of the pBSK_{RNA}-AtRNA plasmid and the resulting RNA was named AtRNA-mala (Fig. 1a).

Sequence encoding for the MS2 coat protein (GenBank: AAA32260.1) was subcloned in the pACYCT2 between the NdeI and XhoI restriction sites. The vectors we describe is derived from pBSTNAV and from the pACYCduet [9]. All the plasmids derived from pACYCduet can be obtained from Addgene. tRNA scaffold vectors derived from pBSTNAV can be obtained from the Laboratory of Biological Crystallography and NMR.

2. As the lpp promoter on the expression vector is constitutive, there is no need for induction. RNA accumulates throughout the culture growth, up to the early stationary phase. Growing cells in rich medium is very important, as the expression of most tRNA processing enzymes is growth rate-dependent and thus tRNA chimera processing is also growth rate-dependent. For the same reason, it is important to use an “efficient” host strain, i.e., not carrying mutations affecting growth rate. Most standard laboratory *E. coli* strains used for protein expression were found to be efficient in this respect, e.g., BL21(DE3), DH5a, JM101, and other derivatives.
3. In order to observe the same background noise, centrifuge 100 μ L of culture per 1 OD₆₀₀ before and after the induction. The pellets will be resuspended in 10 μ L of water and 5 μ L of Laemmli buffer. The samples will be heated for 2 min at 95 °C.
4. Phenol is *toxic and corrosive, so wear gloves and handle under a fume hood*.
5. You can use transparent film for wrapping food or any support UV transparent support that prevents the adsorption of the wet gel on the white paper.
6. You can choose between a batch purification using Ni-NTA Superflow onto a column with capped bottom outlet or a purification on chromatography workstations using Ni-NTA Superflow cartridges.
7. This purification step can be followed by a gel filtration step on a suitable chromatographic medium; typically, Superdex 75 can be used for small constructs (10–40 kDa) and Superdex 200 for larger constructs (50–150 kDa).

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Production of Homogeneous Recombinant RNA Using a tRNA Scaffold and Hammerhead Ribozymes

Frank H.T. Nelissen, Hans A. Heus, and Sybren S. Wijmenga

Abstract

Bacterial overproduction of recombinant RNA using a tRNA scaffold yields large amounts of chimeric RNA. For structural and functional characterizations of the RNA it is often necessary to remove the scaffold. Here we describe an efficient and facile method to release the RNA of interest from the tRNA scaffold by selective cleavage using *cis*-acting hammerhead ribozymes. After cleavage, the RNA of interest is purified to homogeneity using standard chromatographic and electrophoretic methods. Up to 5 mg of highly pure end-product RNA can be obtained from a single liter of bacterial culture.

Key words Recombinant RNA, tRNA scaffold, Hammerhead ribozyme, *Escherichia coli*, RNA purification

1 Introduction

Our protocol for the production of homogeneous recombinant RNA is based on the tRNA scaffold approach that has been first described by Ponchon and Dardel [1–3]. The produced recombinant RNA is a fusion between the RNA of interest and the tRNA scaffold. For structural, functional and pharmacological characterizations and screening it is often desirable to study the RNA of interest without the tRNA scaffold attached. Ponchon and Dardel originally demonstrated the removal of the scaffold with a pair of guide oligonucleotides complementary to the invariable part of the scaffold using RNase H cleavage followed by high-resolution anion exchange purification. As an alternative, Liu et al. [4] employed a production system in which the 5S ribosomal RNA serves as protective scaffold and used biotinylated DNAzyme oligos for the release of the RNA of interest followed by preparative gel purification. To simplify the release from the scaffold described in the above two systems, we designed the recombinant RNA molecules such that the termini of the RNA of interest are flanked with *cis*-acting hammerhead ribozymes (HH) [5, 6]. This enables fast,

efficient, and facile release of the RNA of interest by the addition of Mg^{2+} ions. Upon incubation of diluted crude RNA extract with 10 mM Mg^{2+} at 37 °C, the vast majority of the hammerhead ribozymes self-cleaves in 1 h. There are no additional enzymatic manipulations or DNA oligonucleotides required to release the RNA of interest from the scaffold, making this method a very cost-effective approach to produce large amounts of homogeneous recombinant RNA. Purification of the RNA of interest from the scaffold is performed by anion exchange chromatography and denaturing polyacrylamide gel purification. A careful *in silico* design of the full recombinant RNA molecule to be produced is required since the individual regions of the RNA need to fold in the correct way to retain their biological function. The proposed method is applicable to virtually any RNA molecule of interest [7]. As an example of the method we present the design and production of a recombinant RNA molecule that yields the epsilon replication signal RNA of the Human Hepatitis B Virus (HHBV ϵ) [8, 9].

2 Materials

All solutions need to be prepared using ultrapure water (i.e., deionized water purified to a sensitivity of 18 M Ω cm at 25 °C) and analytical grade reagents. The reagents are prepared and stored at room temperature, unless indicated otherwise. Solutions that need to be sterilized are autoclaved for 15 min at 15 psi on liquid cycle, unless indicated otherwise.

2.1 RNA Production and Extraction

1. pET23b-tRNA-HH₁-hHBV ϵ -HH₂ expression vector for recombinant RNA production (*see* **Note 1**).
2. Competent *E. coli* BL21 (DE3), stored at -80 °C (Novagen) (*see* **Note 2**).
3. Ampicillin stock solution: 100 mg/mL. Weigh out 500 mg of ampicillin sodium salt and dissolve to 5 mL with water and 0.22 μm filter-sterilize. Aliquot in 1.5 mL vials and store at -20 °C.
4. Luria Broth (LB medium) [10]: weigh out 10 g of tryptone, 5 g of yeast extract powder, and 10 g of sodium chloride. Bring to 1 L with demi-water and adjust the pH with 1 M NaOH to 7.0. Sterilize by autoclaving.
5. LB-agar plates: Add 3.75 g of agar to 250 mL of LB medium before autoclaving. After autoclaving, allow the medium to cool to approximately 55 °C and add 125 μL of ampicillin stock solution. Mix by gentle swirling. Then pour the plates of 10–15 mL in a sterile disposable Petri dish ($\varnothing$ 9 cm); use directly or store at 4 °C for up to 2 weeks.

6. 1 M isopropyl β -D-1-thiogalactopyranoside (IPTG) stock solution: weigh out 2.38 g of IPTG and dissolve to 10 mL with water. Aliquot in portions of 1 mL and store at -20°C .
7. Lysis buffer: 10 mM Tris-HCl (pH 7.4), 10 mM MgOAc. Weigh out 1.21 g of Tris and 2.14 g of magnesium acetate tetrahydrate and dissolve to 980 mL with water in a graduated cylinder. Adjust the pH to 7.4 with 3 M HCl and add water to 1 L. Sterilize by autoclaving.
8. Acidic phenol solution: weigh out phenol crystals in a sterile 50 mL Greiner tube and liquefy using lysis buffer (*see Note 3*).
9. Water bath at 42°C .
10. Incubator oven at 37°C .
11. Baffled 2 L shake flask.
12. Temperature controlled orbital shaking incubator.
13. Spectrophotometer at 600 nm (e.g., Ultrospec, GE Healthcare).
14. Centrifugal tubes, 500 mL (Beckman or equivalent).
15. Sterile disposable 50 mL polypropylene (PP) tubes (Greiner or equivalent).

2.2 Hammerhead Cleavage and RNA Purification

1. Dialysis buffer (10 $\times$): 200 mM Tris-HCl (pH 8.1), 2.5 mM EDTA. Weigh out 24.23 g of Tris and 0.93 g of Na₂EDTA and dissolve to 950 mL with water in a graduated cylinder. Adjust the pH to 8.1 with 6 M HCl and add water to 1 L. Sterilize by autoclaving. Dilute tenfold with water prior to use.
2. 20 mM Tris-HCl (pH 8.1): weigh out 2.42 g of Tris and dissolve to 980 mL with water in a graduated cylinder. Adjust the pH to 8.1 with 3 M HCl and add water to 1 L. Sterilize by autoclaving.
3. 1 M MgCl₂ solution: weigh out 20.33 g of MgCl₂·6H₂O and dissolve to 100 mL with water in a graduated cylinder. Sterilize by autoclaving.
4. Column buffer A: 25 mM Tris-HCl (pH 8.1), 1 mM EDTA. Weigh out 3.03 g of Tris and 0.37 g of Na₂EDTA and dissolve to 980 mL with water in a graduated cylinder. Adjust the pH to 8.1 with 3 M HCl and add water to 1 L. Sterilize by autoclaving.
5. Column buffer B: 25 mM Tris-HCl (pH 8.1), 1 mM EDTA, 1.5 M NaCl. Weigh out 3.03 g of Tris, 0.37 g of Na₂EDTA, and 87.66 g of NaCl and dissolve to 980 mL with water in a graduated cylinder. Adjust the pH to 8.1 with 3 M HCl and add water to 1 L. Sterilize by autoclaving.
6. RNA loading buffer (2 $\times$): 5 mL of 0.5 M Na₂EDTA in 45 mL of formamide containing 0.02 % bromophenol blue and 0.02 % xylene cyanol.

7. 40 % acrylamide–bis-acrylamide solution (19:1), store at 4 °C (Merck).
8. Urea.
9. 10 % ammonium persulfate solution in water, store at 4 °C.
10. *N,N,N,N'*-Tetramethyl-ethylenediamine (TEMED), store at 4 °C.
11. TBE electrophoresis buffer (10×): 890 mM Tris, 890 mM H_3BO_3 , 25 mM EDTA. Weigh out 107.8 g of Tris, 55.0 g of H_3BO_3 , and 9.3 g of Na_2EDTA and dissolve to 1 L. Do not adjust the pH; it is 8.3 by itself. Dilute tenfold prior to use.
12. Stains-All staining solution: weigh out 0.16 g of Stains-All (Acros) and bring the powder in a 1 L amber bottle. Add 560 mL of formamide and dissolve the Stains-All by swirling. Add 440 mL of water and mix well. Store in the dark.
13. 3 M NaOAc (pH 5.2): weigh out 24.61 g of anhydrous sodium acetate and dissolve to 70 mL in a graduated cylinder. Adjust the pH to 5.2 by adding 100 % acetic acid and add water to 100 mL. Sterilize by autoclaving.
14. Ethanol.
15. High salt dialysis buffer: 20 mM sodium phosphate buffer (pH 7.5), 1 M NaCl, 50 mM EDTA. Combine 40 mL of 50 mM sodium phosphate buffer (pH 7.5), 20 mL of 5 M NaCl, 10 mL of 0.5 M EDTA (pH 8.1), and 20 mL of water. pH adjustment is not necessary. Sterilize by autoclaving.
16. Dialysis tubing (Spectra, 6–8 kDa MWCO, or equivalent).
17. Centrifugal tubes, 250 mL (Beckman or equivalent).
18. ResourceQ 6 mL anion exchange column (GE Healthcare) or equivalent.
19. ÄKTA FPLC system (GE Healthcare) or equivalent.
20. Vertical gel electrophoresis system for 17 × 15 cm ($W \times L$) gels (Biometra Model V15.17 or equivalent).
21. Glass plates, 0.8 mm spacers and a 14-well comb for analytical gel electrophoresis (Biometra or equivalent).
22. Light-tight container.
23. Developer tray.
24. Vertical gel electrophoresis system for 31 × 38.5 cm ($W \times L$) gels (Biometra Model S2 or equivalent).
25. Glass plates, 3 mm spacers and a single-well comb for preparative gel electrophoresis (Biometra or equivalent).
26. Syringe with 19 G needle.
27. Metal plate (ca. 250 × 150 × 1 mm).
28. Scalpel.
29. TLC Silica gel 60F₂₅₄ plates (Merck or equivalent).

30. Household foil.
31. Handheld UV lamp.
32. Elutrap device + membranes for electro elution (Schleicher & Schuell or equivalent).
33. Centrifugal ultrafiltration units (Centricon YM-10 or equivalent, Millipore).

3 Methods

3.1 Design of the Recombinant RNA Molecule

A proper *in silico* design of the molecule is an important aspect for successful recombinant RNA production. The elements that are present in the RNA molecule have to fold correctly and, simultaneously, should not disturb the fold of the full molecule. Guided by the secondary structure prediction of the construct for HHBV ε -RNA production displayed in Fig. 1a, we briefly provide a description for the design principles of such a recombinant RNA molecule.

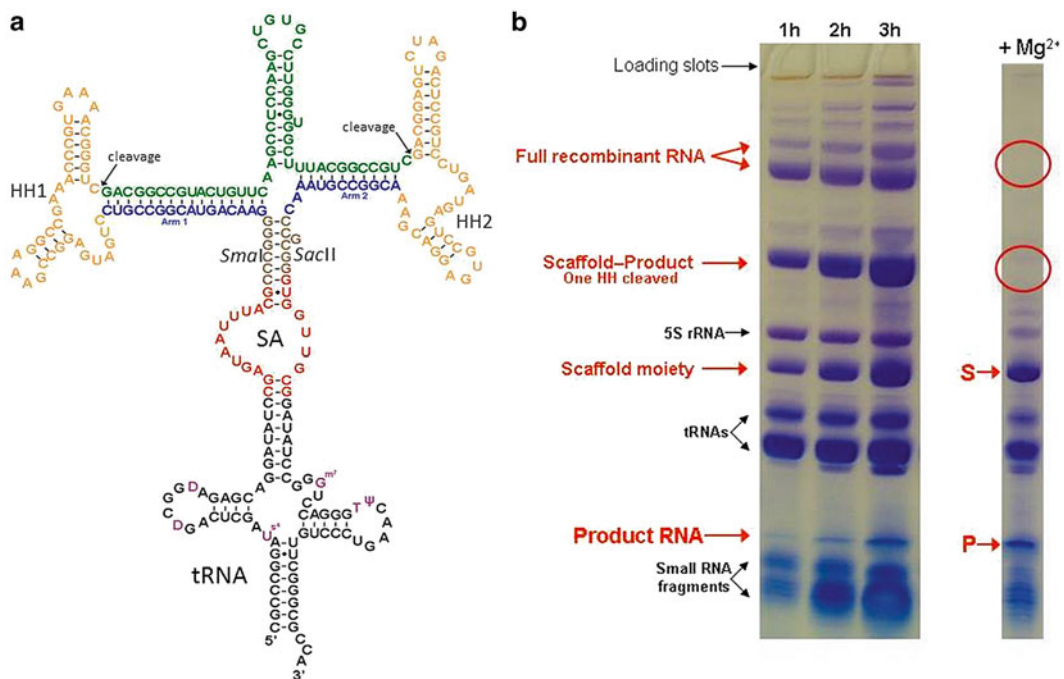


Fig. 1 (a) Predicted secondary structure of the full recombinant RNA for the production of the 53 nt HHBV ε -RNA. The HHBV ε -RNA is located in between the two HH cleavage sites that are indicated with arrows. The cassette is placed onto the tRNA scaffold containing the Sephadex G-100 aptamer (SA) and the cloning site (SmaI/SacII), generating the 4-way junction. (b) Course of a large-scale recombinant RNA production. Samples of 1 mL were withdrawn from the culture at respectively 1 h, 2 h and 3 h post induction and processed for analytical polyacrylamide gel analysis (see Note 7). After addition of $MgCl_2$ for complete self-cleavage of the HH ribozymes, the full recombinant RNA and the partially cleaved recombinant RNA disappear and only the scaffold (S) and the released product (P) remain visible as recombinant RNA

1. Write down (horizontally, Fig. 1a) the first 10–15 nucleotides from the 5'-end of the RNA of interest and place the complementary nucleotides below (Arm 1, Fig. 1a).
2. Perform the same for the last 10–15 nucleotides at the 3'-end of the RNA of interest (Arm 2, Fig. 1a).
3. Place the remainder of the nucleotides of the RNA of interest on top in the middle (Fig. 1a).
4. Place the sequence of hammerhead ribozyme 1 (HH1) in front of the 5'-end of the RNA of interest and place hammerhead ribozyme 2 (HH2) at the 3'-end (Fig. 1a and *see* **Note 4**).
5. This whole cassette can now be placed on top of the tRNA scaffold containing a Sephadex G-100 aptamer sequence [**11**] (*see* **Note 5**).
6. Use the RNA folding program on the Mfold webserver [**12**, **13**] to simulate the fold of the constructed RNA molecule (do not use constraints and do not change the standard folding conditions).
7. Make changes in the nucleotides of the arms complementary to the RNA of interest in the 4-way junction region (i.e., junction between product, both arms and the scaffold) until the desired fold is predicted to be more stable than alternative folds (*see* **Note 6**).
8. Synthesize the desired DNA sequence using partially overlapping DNA oligonucleotides or order it as a whole from a company (*see* **Note 7**).

3.2 RNA Production and Extraction

1. Transform 200 μ L of competent *E. coli* BL21 (DE3) cells with 25–250 ng of pET23b-tRNA-HH₁-hHBV ϵ -HH₂ expression vector.
2. Spread 25–100 μ L on a LB-agar plate containing 50 μ g/mL ampicillin and incubate overnight at 37 °C.
3. Next morning, pick a single colony and cultivate in 5 mL of LB medium containing 50 μ g/mL ampicillin (LB-amp⁵⁰) at 37 °C, 225 rpm for 8 h.
4. Transfer 300 μ L of this culture to 30 mL of LB-amp⁵⁰ and cultivate overnight at 37 °C, 225 rpm.
5. Use the overnight culture to inoculate 1 L of LB-amp⁵⁰ in a baffled 2 L shake flask.
6. Shake the flask at 37 °C, 225 rpm until the culture reaches an OD₆₀₀ of ~0.6–0.8.
7. Initiate recombinant RNA production by adding 1 mL of 1 M IPTG (final concentration 1 mM) and continue shaking for an additional 3–4 h (*see* **Note 8** and Fig. 1b).
8. Harvest the cells by centrifugation for 10 min at 5,000 $\times g$, 4 °C.

9. Resuspend the cells in 40 mL lysis buffer and divide into two sterile 50 mL disposable polypropylene tubes.
10. Add 10 mL of saturated phenol per tube and agitate gently for 30 min at room temperature.
11. Separate phases by centrifugation at $7,500\times g$, 4 °C for 10 min (*see Note 9*).
12. Pipette off the aqueous supernatant and repeat the extraction procedure with another 10 mL of lysis buffer per tube.
13. Pool the individual supernatants and keep at 4 °C.

3.3 Hammerhead Ribozyme Cleavage and RNA Purification

1. Bring the supernatants in a 6–8 kDa MWCO dialysis tube and dialyze against 2.5 L of dialysis buffer at 4 °C for at least 4 h.
2. Replace the dialysis buffer and repeat the dialysis step.
3. Dilute the dialyzed RNA fourfold with 20 mM Tris–HCl (pH 8.1) and add sterile MgCl_2 stock solution to a final concentration of 10 mM.
4. Incubate at 37 °C for 1 h.
5. Remove formed precipitate by centrifugation at $20,000\times g$, 4 °C for 30 min (*see Note 10*).
6. Connect the ResourceQ 6 mL column to the ÄKTA FPLC and equilibrate with five column volumes (CV) of Column buffer A at a flow rate of 5 mL/min.
7. Load the supernatant onto the column and apply a segmented gradient elution with Column buffer B to elute the bound RNA (*see Note 11* and Fig. 2a).
8. Collect fractions of 5 mL.
9. Take 20 μL of each fraction and mix with 20 μL of 2 $\times$ RNA loading buffer, boil at 100 °C for 2 min.
10. Prepare an 8 % gel solution in a sterile 500 mL Erlenmeyer by mixing 100 mL of 40 % acrylamide–bis-acrylamide solution (19:1), 50 mL of 10 $\times$ TBE and 240.5 g of urea. Bring the volume to 500 mL with water.
11. Assemble the glass plates of an analytical gel (17 $\times$ 15 cm) using spacers of 0.8 mm thick. Add 100 μL of 10 % APS and 40 μL of TEMED to 25 mL of the gel solution, mix well and pour between the glass plates. Directly insert a 14-well comb (well dimensions ($W\times L$): 8 $\times$ 15 mm) without introducing air bubbles.
12. Allow the gel to polymerize for an hour, assemble the gel in the electrophoresis system and pre-electrophorize for 15 min at 500 V in 1 $\times$ TBE (*see Note 12*).
13. Load 25 μL of each sample on the gel and electrophorize at 500 V until the bromophenol blue front reaches the bottom of the gel.

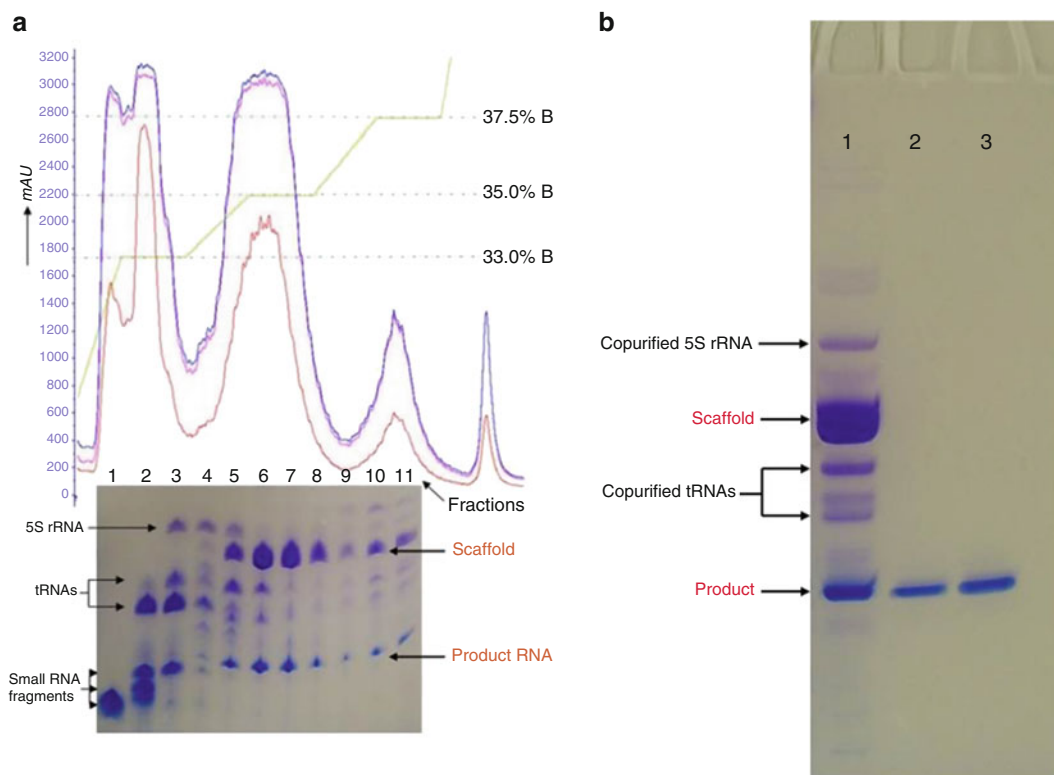


Fig. 2 (a) FPLC anion exchange elution profile of a HH cleaved recombinant RNA extract from 1 L of culture. UV absorbance is measured at 260 nm, 254 nm (*upper* two traces) and 280 nm (*lower* trace). The analyzed samples on the polyacrylamide gel below the elution profile correspond to the samples taken from the collected fractions during purification (*see* Subheading 3.3, **step 9**). The percentages of Column buffer B are indicated with *dashed lines* and show the elution of the different species at increasing salt concentration. **(b)** Lane 1 contains 30 µg of anion exchange column purified RNA. Lanes 2 and 3 show the final purity of respectively 2 and 4 µg of preparative denaturing polyacrylamide gel purified RNA

14. Carefully pry the glass plates open with a stiff spatula and immerse the gel for 45 min in a light-tight container containing Stains-All staining solution.
15. Immerse the gel in water in a developer tray and destain by exposing the gel to (day) light until the purple background has disappeared.
16. Pool the fractions that contain the recombinant RNA product (*see* **Note 13** and Fig. 2a).
17. Add 1/10 volume of 3 M NaOAc (pH 5.2) and subsequently 3 volumes of 100 % ethanol. Precipitate the RNA at -20°C , preferably overnight.
18. Collect the precipitated RNA by centrifugation at $20,000\times g$ for 1 h.
19. Decant the supernatant, reconstitute the pellet in 2 mL water and mix with an equal volume of $2\times$ RNA loading buffer for RNA (take the increase in volume into account).

20. Boil the sample for 10 min at 100 °C.
21. Assemble the glass plates for a preparative gel (31 × 38.5 cm) using spacers of 3 mm thick. Add 200 µL of 10 % APS and 80 µL of TEMED to 50 mL of the gel solution, mix well and pour between the glass plates to seal the bottom of the gel. Slightly tilt the gel to ensure the gel solution is at the bottom of the assembly.
22. Allow the gel to polymerize for 45 min and then add 1 mL of 10 % APS and 250 µL of TEMED to 400 mL of gel solution. Mix well and pour on top of the sealing gel.
23. Directly insert a single-well comb and now allow the gel to polymerize horizontally for 2 h.
24. Assemble the gel in the electrophoresis system and pre-electrophorize in 1× TBE buffer for 30 min at 300 V.
25. Carefully rinse the urea from the large well and load the sample onto the gel (*see Note 12*).
26. Electrophorize overnight at 300 V.
27. Carefully pry the glass plates open using a metal plate of 1 mm thick (*see Note 14*).
28. Put the gel onto a TLC Silica gel 60F₂₅₄ plate that is wrapped in plastic household foil.
29. Put the gel under a handheld UV lamp and excise the RNA of interest from the gel using a scalpel.
30. Cut the gel band into pieces of approximately 5 cm and elute the RNA from the gel slices into sterile TBE at 180 V using an Elutrap device according to the manufacturer's instructions (*see Note 15*).
31. Collect the RNA from the Elutrap and recover the RNA by ethanol precipitation as described in **steps 17–19** (*see Fig. 2b*).
32. Reconstitute the RNA in 500 µL of water and dialyze using centrifugal ultrafiltration against high salt dialysis buffer in a Centricon YM-10 device (or equivalent) and subsequently against sterilized water.
33. Use the purified RNA by adding buffer salts of choice, store the RNA at –20 °C or lyophilize the isolate for long-term storage.

4 Notes

1. The pET23b plasmid for recombinant RNA synthesis can be obtained from the Laboratory of Biophysical Chemistry, Radboud University, Nijmegen, The Netherlands.

2. The recombinant RNA production relies on transcription by bacteriophage T7 RNA polymerase. The bacterial host should therefore contain a chromosomal insertion of the T7 RNA polymerase gene to enable T7 promoter-driven RNA transcription.
3. Make sure the liquefied phenol is acidic by dipping a clean pH strip in it. Do not omit the MgOAc since this would result in the co-extraction of the large subunits of the ribosomal RNA into the aqueous phase. Phenol is preferably weighed out and handled wearing protective clothing, glasses, and gloves in a hood because of its toxicity and pungent smell.
4. Be aware that the catalytic core of the Hammerhead ribozyme contains several conserved nucleotides that are required for activity [5]. For the ribozyme at the 5'-end of the RNA of interest (HH1) there are no difficulties implementing the sequence, since the ribozyme cleaves itself after the consensus sequence UX-3' (where X ≠ G). At the 3'-end of the RNA of interest, however, the helices of the ribozyme (HH2) are insurmountably different in orientation and therefore require the penultimate nucleotide of the RNA of interest to be a U-residue and the terminating residue any residue but a G. We have chosen to produce the RNAs of interest so that they yield closed stems (5'GAC~~~GUC3') at the termini. A potential way to circumvent this sequence dependence at the 3'-end of the RNA of interest could be the implementation of a hairpin ribozyme [14, 15] instead of a hammerhead ribozyme.
5. The RNA stem on top of the Sephadex G-100 aptamer sequence contains a *Sma*I (5') and a *Sac*II (3') restriction site that can be used for subcloning on DNA level of different cassettes containing RNAs of interest.
6. The 4-way junction region can be altered by changing the length of the arms and/or introducing unpaired nucleotides in the junction. We usually try not to make the fold in the junction too tight, i.e., there are a few unpaired nucleotides present that allow for some flexibility in the fold of the molecule. A positive effect of unpaired nucleotides at the junction is supported by the production of the guanine riboswitch aptamer domain [16]. It did not produce very large amounts when employing the published sequence [7], but after introducing two extra unpaired nucleotides in the lower region of the junction the yield became comparable to that of the other RNAs (unpublished results). A similar positive effect was found for the yield of the TAR element of HIV-1 [17]. Furthermore, the length of the RNA of interest should differ by at least ten nucleotides from the scaffold moieties to prevent difficulties during preparative denaturing gel purification.
7. Make sure that the correct restriction sites are present for subcloning of the fragments. RNA cassettes that are to be cloned

on top of the scaffold+Sephadex G-100 aptamer should contain *Sma*I at the 5'-end and *Sac*II at the 3'-end. The full recombinant molecule that is placed in the pET23b vector should be flanked with *Xba*I at the 5'-end (shortly after the vector-borne T7 promoter and a site from the multiple cloning site (we used *Xba*I)). We successfully used pET28a as expression vector alternatively to pET23b; however, using pUC18 yielded no significant amount of recombinant RNA.

8. We usually take samples of 1 mL of culture every hour post-induction for checking the RNA transcription level of a large culture. Spin down the cells and store the pellet on ice, resuspend in 100 μ L of lyses buffer, extract with 100 μ L of phenol saturated with lysis buffer, and collect the aqueous phase. Ethanol precipitate as described in Subheading 3.3 steps 17 and 18, reconstitute in 20 μ L of water and add 20 μ L of 2 $\times$ LB for RNA. Analyze on analytical denaturing PAGE as described in Subheading 3.3 steps 11–15 (see Fig. 1b).
9. Do not spin faster to prevent breakage of the tubes. The aqueous phase might occur as a whitish suspension. Try to pipette off as much as possible, without disturbing the thick white interface that is present between the organic phase and aqueous phase.
10. The presence of a precipitate might not be visible by eye. However, to prevent clogging of the column this step cannot be circumvented. Alternatively, 0.22 μ m filter-sterilize the solution.
11. We applied a segmented linear gradient of 10 CV (CV stands for column volume) from 0 % B to 33 % B (*X*% B stands for *X*% of Column buffer B) $\rightarrow$ 2 CV at 33 % B $\rightarrow$ 2 CV to 35 % B $\rightarrow$ 2 CV at 35 % $\rightarrow$ 2 CV to 37.5 % $\rightarrow$ 2 CV at 37.5 % B $\rightarrow$ 5 CV to 100 % B. This gives in our hands the optimal separation of different RNA species.
12. Rinse the wells with 1 $\times$ TBE to remove urea using a syringe with a needle. The thick layer of urea otherwise prohibits the RNA to migrate into the gel efficiently.
13. RNA fractions that contain a relatively large amount of tRNA and/or degradation fragments are excluded from the recombinant RNA pool. Their presence might complicate the subsequent preparative denaturing polyacrylamide gel purification.
14. Do not make use of a spatula to pry open the large glass plates. They are closed very tightly and most likely the plates will break or splinter.
15. Electro elution gives reproducible yields in our hands, although care has to be taken not to introduce contaminations during this step (e.g., use sterile TBE). Methods such as RNA elution by crushing and soaking [18] the gel fragments can be employed alternatively as a cost efficient method.

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In Vivo Production of Small Recombinant RNAs Embedded in a 5S rRNA-Derived Protective Scaffold

Victor G. Stepanov and George E. Fox

Abstract

Preparative synthesis of RNA is a challenging task that is usually accomplished using either chemical or enzymatic polymerization of ribonucleotides in vitro. Herein, we describe an alternative approach in which RNAs of interest are expressed as a fusion with a 5S rRNA-derived scaffold. The scaffold provides protection against cellular ribonucleases resulting in cellular accumulations comparable to those of regular ribosomal RNAs. After isolation of the chimeric RNA from the cells, the scaffold can be removed if necessary by deoxy-ribozyme-catalyzed cleavage followed by preparative electrophoretic separation of the cleavage reaction products. The protocol is designed for sustained production of high quality RNA on the milligram scale.

Key words Recombinant RNA, In vivo RNA production, 5S rRNA-derived scaffold

1 Introduction

The intracellular concentration of a recombinant RNA expressed in a bacterial host depends heavily on its resistance to the host nucleic acid degradation machinery. In the *Escherichia coli* cytoplasm, the recombinant RNA may become a target for about 20 different ribonucleases (RNases), which are normally involved in maintenance and regulation of mRNAs turnover, maturation and quality control of noncoding RNAs, and anti-phage defense [1–4]. To decrease the adverse effect of RNases on yield, the RNA of interest can be expressed as a fusion with a stable cellular RNA that plays a role of a protective scaffold. When inserted into the protective scaffold, the recombinant RNA becomes unsusceptible to polyadenylation-mediated digestion by the degradosome, and to exoribonuclease attack on its termini. It may also become less prone to cleavage if the scaffold sterically hinders endoribonuclease binding to the recombinant RNA insert. After isolation of the expressed chimera from the cells, the inserted RNA can be released from the protective scaffold by treatment with an appropriate RNA-cleaving enzyme.

The protocol described here uses a 5S rRNA-derived protective scaffold for the in vivo production of large quantities of recombinant RNAs. Earlier, it was found that a plasmid borne deletion mutant of *Vibrio proteolyticus* 5S rRNA devoid of helix III and loop C continued to be expressed in *E. coli*, but did not enter the ribosomes [5]. A small artificial RNA, *Bst* aRNA, was created on the base of this mutant by introducing a *Bst*EII restriction site in its coding sequence. This allows easy insertion of extension modules in place of the deleted fragment [5] (Fig. 1a, b). The *Bst* aRNA accumulated in the cells at levels similar to the native 5S rRNA (in excess of 10,000 copies per cell) without incorporating into the ribosomes. The expression efficiency was similar for the *Bst* aRNA expression cassette placed in either the plasmid or the bacterial chromosome [6]. Transcriptome studies revealed that the *Bst* aRNA does not cause a significant metabolic disturbance in the host [7]. This is consistent with the fact that 5S rRNA per se is neither a regulator nor a subject of direct regulation [8], and is not involved in any major cellular process outside of the translation apparatus. Overall, the *Bst* aRNA was apparently suitable for the role of an expression vehicle for short RNA inserts. The utility of *Bst* aRNA was confirmed by the expression in *E. coli* of *Bst* aRNA-embedded aptamers [9], recognition tags [5, 10], and randomized libraries for selection experiments [11].

While some of the recombinant RNAs may be used without their release from the protective scaffold, more generally it is necessary to cut them out. To accomplish this, the chimeric RNA can be treated with a pair of deoxyribozymes (DNAzymes) [12]. After completion of the cleavage reaction, the released recombinant RNA can be separated from the remains of the protective scaffold and the DNAzymes by preparative electrophoresis in denaturing polyacrylamide gel. The procedure can be further improved through the use of biotinylated DNAzymes (Fig. 1c). The presence of a biotin tag allows removal of the DNAzymes from the cleavage reaction mixture by affinity capture on streptavidin beads. This decreases the gel load and makes it easier to recover the DNAzymes for reuse. Overall, the procedure yields sufficient quantities of highly homogeneous RNA suitable for various downstream applications.

2 Materials

2.1 Bacterial Strain and Plasmid Vector

1. *Escherichia coli* strain JM109 [endA1 glnV44 thi-1 relA1 gyrA96 recA1 mcrB⁺ Δ(lac-proAB) e14-[F' traD36 proA⁺B⁺ lacI^q lacZΔM15] hsdR17(r_K⁻ m_K⁺)] (New England Biolabs) is used as a host for RNA expression vector (*see Note 1*). Electrocompetent cells, suspension in 10 % glycerol. Store at (-80)°C.

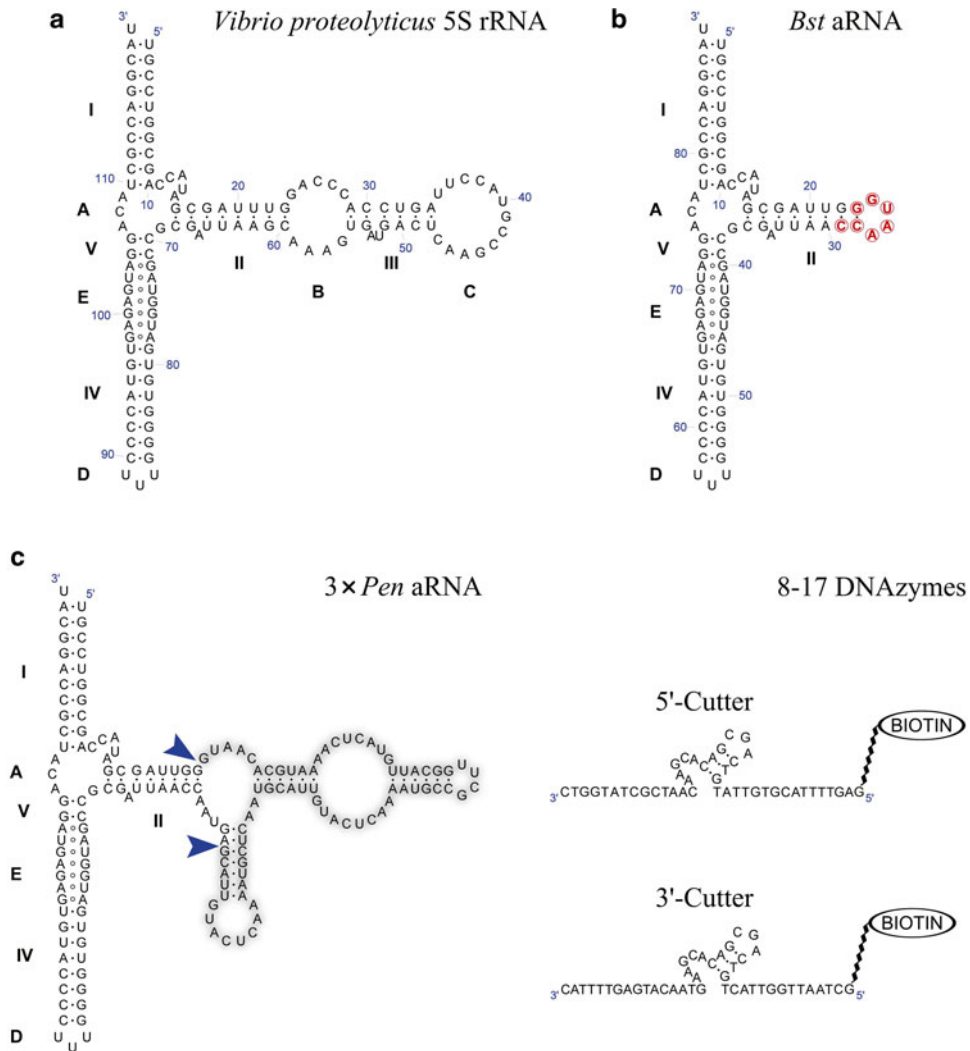


Fig. 1 Sequence and predicted secondary structure of *V. proteolyticus* 5S ribosomal RNA and the derived artificial RNAs. Helices are indicated by *Roman numerals*, and loops are indicated by *capital letters* according to the current nomenclature of 5S rRNA secondary structure elements. Base pairing in “loop E” is shown by *open circles*. **(a)** Native *V. proteolyticus* 5S rRNA. **(b)** *Bst* aRNA (88 nt), a stable artificial RNA obtained by deletion of helix III and loop C in native *V. proteolyticus* 5S rRNA. Nucleotides corresponding to *Bst*EII restriction site in the coding sequence of *Bst* aRNA are *circled*. **(c)** $3 \times \textit{Pen}$ aRNA (160 nt), a chimeric RNA obtained by fusion of identification tag for *Pennisetum purpureum* (shown as *shadowed sequence*) with *Bst* aRNA, and a pair of DNAzymes used for cutting out the tag after isolation and purification of the chimeric RNA. The cleavage sites are indicated by *arrows*

2. pCA2c plasmid vector carrying an artificial operon for constitutive expression of *Bst* aRNA, a small RNA derived from *Vibrio proteolyticus* 5S rRNA by removing helix III-loop C segment and by introducing a *Bst*EII restriction site in the coding sequence of the loop B (*see Note 2*) (Fig. 2). Aqueous solution, 0.1 mg/mL. Store at $(-80)^{\circ}\text{C}$.

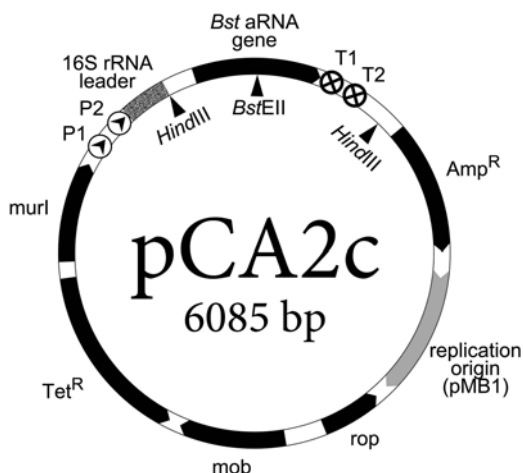


Fig. 2 Map of the pCA2c plasmid carrying *Bst* aRNA-expressing cassette. The cassette is derived from *E. coli* rrnB operon. It contains rrsB P1 and P2 promoters followed by the leader sequence of the 16S rRNA gene, *Bst* aRNA coding sequence, and T1 and T2 transcription terminators. Besides the *Bst* aRNA-expressing cassette, the plasmid contains genes for beta-lactamase (Amp^R), Rop protein (rop), Mob-like protein (mob), tetracycline resistance protein (Tet^R), and glutamate racemase (murl)

2.2 Equipment

1. Thermal cycler with temperature-controlled lid.
2. Electroporator capable to deliver 5 ms-long pulse with 1.4–1.6 kV output.
3. Thermostatic incubator with shaking platform set at 37 °C and 250 rpm.
4. UV transilluminator.
5. Vertical gel electrophoresis system with glass plates 20 × 20 cm.
6. Horizontal gel electrophoresis system.
7. Power supply capable to produce up to 600 V output.
8. Tabletop centrifuge capable to deliver centrifugal force of at least 10,000 × *g* with fixed-angle rotor for 1.5 mL microcentrifuge tubes.
9. Low-speed centrifuge with swinging-bucket rotor for 15 mL Falcon tubes.
10. Refrigerated centrifuge capable to deliver centrifugal force of at least 10,000 × *g* with fixed-angle rotors for 15, 50 and 250 mL centrifuge tubes.
11. Tabletop thermostatic incubator.
12. UV spectrophotometer.

2.3 Supplies

1. PCR tubes, 0.2 mL, sterile.
2. Microcentrifuge tubes, 1.5 mL, sterile.

3. Falcon tubes, 5, 15, and 50 mL, sterile.
4. Polypropylene centrifuge tubes, 250 mL.
5. Electroporation cuvette with 0.1 cm gap, sterile.
6. Spin columns with silica membrane, 0.6 mL bed volume (*see Note 3*).
7. Petri dishes (100 × 15 mm), sterile.
8. Spectrophotometric cuvettes, UV-transparent.
9. Wooden toothpicks, sterile.
10. Dialysis membrane with 600 Da pore size, autoclaved. Store in sterile water at room temperature.
11. Erlenmeyer flask, 2.5 L.
12. Thin-layer chromatographic plate with fluorescent indicator, 20 × 20 cm.
13. Razor blades.
14. Saran wrap.

2.4 Reagents

All solutions are prepared using ultrapure water with resistance of 17–18 MΩ except for bacterial growth media that can be made using deionised water (*see Note 4*). The reagents are stored at 4 °C unless otherwise mentioned.

2.4.1 General Purpose Reagents

1. Sodium Acetate Buffer: 3 M sodium acetate, pH 5.0.
2. Ethanol, 96 % or absolute. In the protocol it is referred to as simply ethanol.
3. Ethanol, 70 %.
4. Tris–EDTA (TE) Buffer: 10 mM Tris–HCl (pH 7.5), 1 mM EDTA–NaOH (pH 8.0).

2.4.2 Cloning of the Recombinant RNA Coding Sequence into pCA2c Plasmid Vector

1. Double-stranded DNA with coding sequence of the recombinant RNA of interest flanked with spacers if necessary (*see Subheading 3.1*) and *Bst*EII restriction sites. The dsDNA can be either obtained commercially or synthesized by overlapping PCR from shorter single-stranded deoxyoligonucleotides. Store at (–20)°C.
2. *Bst*EII restriction endonuclease (New England Biolabs, #R0162, 10,000 units/mL). Store at (–20)°C.
3. 10× NEBuffer 3: 0.5 M Tris–HCl, 1 M NaCl, 0.1 M MgCl₂, 10 mM dithiothreitol, pH 7.9. Store at (–20)°C.
4. Hind III restriction endonuclease (New England Biolabs, #R0104, 20,000 units/mL). Store at (–20)°C.
5. 10× NEBuffer 2: 0.1 M Tris–HCl, 0.5 M NaCl, 0.1 M MgCl₂, 10 mM dithiothreitol, pH 7.9. Store at (–20)°C.

6. T4 DNA ligase (New England Biolabs, #M0202, 400,000 units/mL). Store at $(-20)^{\circ}\text{C}$.
7. 10 $\times$ T4 DNA Ligase Reaction Buffer: 0.5 M Tris-HCl (pH 7.5), 0.1 M MgCl_2 , 0.1 M dithiothreitol, 10 mM ATP. Store at $(-20)^{\circ}\text{C}$.
8. Calf Intestinal Alkaline Phosphatase (New England Biolabs, #M0290, 10,000 units/mL). Store at $(-20)^{\circ}\text{C}$.
9. Neutral Phenol: Phenol saturated with 50 mM Tris-HCl, pH 7.5 (*see* **Note 5**).
10. Chloroform (*see* **Note 6**) mixture. Store at room temperature.
11. 30 % Glycerol. Sterilize by autoclaving.

2.4.3 Plasmid Purification

1. Alkaline Lysis Buffer: 0.2 N NaOH, 1 % (w/v) Sodium dodecylsulfate (SDS). Store at room temperature.
2. Neutralization Buffer: 4.5 M guanidine-HCl, 0.24 M acetic acid, 20 mM EDTA, 20 % ethanol. Neutralization Buffer is prepared on the day of use by mixing 4 volumes of component A with 1 volume of component B. Component A: 5.625 M guanidine-HCl, 0.3 M acetic acid, 25 % ethanol. Component B: 0.1 M EDTA-NaOH (pH 8.0). Both components A and B can be stored at room temperature.
3. Salt Wash Buffer: 5 M guanidine isothiocyanate, 0.1 M EDTA-NaOH (pH 8.0). Store at room temperature protected from light.
4. Ethanol Wash Buffer: 75 % ethanol, 10 mM Tris-HCl (pH 7.5). Store at room temperature.

2.4.4 Bacterial Cells Growth

1. Ampicillin 1,000 $\times$ Stock: ampicillin, sodium salt, 100 mg/mL solution in water, filter-sterilized. Store at $(-20)^{\circ}\text{C}$.
2. LB Medium: 10 g/L Bacto-Tryptone, 5 g/L yeast extract, 10 g/L NaCl, adjust pH to 7.2 with NaOH, autoclave.
3. LB agar plates with ampicillin: 10 g/L Bacto-Tryptone, 5 g/L yeast extract, 10 g/L NaCl, 15 g/L agar. Autoclave, cool down to approximately 50°C while stirring occasionally, add Ampicillin to final concentration 0.1 mg/mL, mix well, and pour into petri dishes. Allow agar to solidify, then air-dry the surface for 1–2 h in aseptic environment. Store in sealed plastic bag.
4. SOC Medium: 20 g/L Bacto-Tryptone, 5 g/L yeast extract, 0.6 g/L NaCl, 0.5 g/L KCl, 10 mM MgCl_2 , 10 mM MgSO_4 , 20 mM glucose. Dissolve Bacto-Tryptone, yeast extract, NaCl, and KCl in water to the final volume 980 mL, autoclave, cool down to room temperature. Prepare 2 M glucose solution and sterilize by filtration. Prepare 1 M MgCl_2 + 1 M MgSO_4 solution, sterilize by filtration. Store the solutions separately.

Immediately prior use, add 10 mL of 2 M glucose and 10 mL of 1 M MgCl_2 + 1 M MgSO_4 to the main broth.

5. Culture Diluent: 0.3 M KCl, 50 mM boric acid.

2.4.5 Cell Lysis and Fractionation of Nucleic Acids

1. Tris–Acetate–EDTA Buffer for Cell Lysis (TAE-CL): 1 M Tris–Acetate, 0.1 M Na_2EDTA , pH 7.5.
2. Formamide (deionized).
3. 10 % (w/v) sodium dodecyl sulfate (SDS). Store at room temperature.
4. Potassium Acetate Buffer: 3 M potassium acetate, 2 M acetic acid. pH value does not have to be precisely set but after mixing all of the components should be approximately 5. Store at room temperature.

2.4.6 Preparative Electrophoresis in Denaturing Polyacrylamide Gel

1. 10× Tris–Borate–EDTA (TBE) Buffer: 0.5 M Tris base, 0.5 M boric acid, 10 mM Na_2EDTA , pH 8.3 (*see Note 7*). Store at room temperature in plastic flask.
2. 1× Tris–Borate–EDTA (TBE) Buffer: 50 mM Tris base, 50 mM boric acid, 1 mM Na_2EDTA , pH 8.3 (*see Note 7*). Store at room temperature in plastic canister.
3. Acrylamide Monomers: 8.17 % (w/v) acrylamide, 0.43 % (w/v) N,N' -methylenebisacrylamide, 7.8 M urea (*see Note 8*). Dissolve urea in warm (~50 °C) water, cool down to room temperature, add dry acrylamide and N,N' -methylenebisacrylamide, stir until completely dissolved. As an optional step, deionize the solution by adding mixed-bed ion-exchange beads (2 g/L), stirring for 3–4 h at room temperature, and filtering the beads out. Store protected from light.
4. Ammonium persulfate, 100 mg/mL. Store protected from light. Once made, the solution can be kept for 2–3 weeks without noticeable loss of activity.
5. N,N,N',N' -tetramethylethylenediamine (TEMED). Store protected from light.
6. 2× Polyacrylamide Gel Loading Buffer with Formamide: 96 % deionized formamide, 20 mM EDTA–NaOH (pH 7.5), 0.04 % (w/v) bromophenol blue, 0.04 % (w/v) xylene cyanol.
7. 0.2× Tris–Acetate–EDTA (TAE) Buffer: 40 mM Tris base, 18 mM acetic acid, 1 mM Na_2EDTA , pH 8.2.

2.4.7 Agarose Gel Electrophoresis

1. Agarose.
2. 1× Tris–Borate–EDTA (TBE) Buffer: 50 mM Tris base, 50 mM boric acid, 1 mM Na_2EDTA , pH 8.3 (*see Note 9*). Store at room temperature in plastic canister.
3. 6× Agarose Gel Loading Buffer: 15 % (w/v) Ficoll 400, 6 mM EDTA–NaOH (pH 7.5), 0.1 % (w/v) Bromophenol Blue.

4. Ethidium bromide, 5 mg/mL. Store protected from light.
5. Hi-Lo DNA ladder (Bionexus, #BN2050).

2.4.8 RNA Cleavage with DNAzymes

1. Biotinylated 5'-Cutter DNAzyme deoxyoligonucleotide, 0.2 mM solution in water. Store at $(-20)^{\circ}\text{C}$ (*see Note 9*).
2. Biotinylated 3'-Cutter DNAzyme deoxyoligonucleotide, 0.2 mM solution in water. Store at $(-20)^{\circ}\text{C}$ (*see Note 9*).
3. MOPS Buffer: 1 M MOPS-NaOH, pH 7.2.
4. 4 M sodium chloride (NaCl). Store at room temperature.
5. 1 M potassium chloride (KCl). Store at room temperature.
6. 0.1 M magnesium chloride (MgCl_2).
7. 0.1 M manganese chloride (MnCl_2).
8. 10 mM spermine hydrochloride. Store aliquoted at $(-80)^{\circ}\text{C}$.
9. Tris-EDTA-NaCl (TEN) Buffer: 15 mM Tris-HCl, 5 mM Na_2EDTA , 0.6 M NaCl, pH 7.5. Store at room temperature.
10. Streptavidin Agarose, sedimented bead suspension (Molecular Probes—Invitrogen, #S951), 50 % slurry of streptavidin-carrying 4 % beaded crosslinked agarose in 100 mM sodium phosphate, 100 mM NaCl, 2 mM NaN_3 , pH 7.5.

2.5 Software

Design of the chimeric RNA is performed using any program or Web-service from the list below:

1. RNAstructure, stand-alone program, freely available at <http://rna.urmc.rochester.edu/RNAstructure.html>.
2. RNAdraw, stand-alone program, freely available via <http://www.rnadraw.com>.
3. Mfold, Web-server, <http://mfold.rna.albany.edu/?q=mfold>.
4. RNAfold, Web-server, <http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi>.

3 Methods

3.1 Guidelines for Designing the Chimeric RNA and DNAzymes

When designing the chimeric RNA, it should be kept in mind that if the cargo RNA sequence exhibits significant complementarity to any part of the protective scaffold, it might prevent the chimera from forming the 5S rRNA-like structure. This in turn may interfere with the correct processing of chimera-encompassing primary transcript, or compromise the protective properties of the scaffold. Also, if the cargo RNA contains exposed long helices or large loose loops, it may be inherently vulnerable to endonuclease attack, and thus deteriorate the cytoplasmic stability of the chimera as a whole. To eliminate undesirable structural motifs brought with or induced by the cargo RNA, one can incorporate additional spacer sequences

between the cargo RNA and the scaffold to force the cargo RNA into more favorable conformation. Creating an alternative base pairing pattern by manipulation with spacer sequences allows one to control the structure of the cargo RNA insert without modifying its sequence. The spacers should be minimally structured in their parts immediately adjacent to the DNAzyme cleavage sites to decrease a competition between DNAzyme binding and intramolecular base pairing.

Base pairing in the designed chimeric RNA can be validated using a secondary structure prediction program RNAdraw or RNAstructure, or an equivalent web-service. The program should be configured to allow for noncanonical base pairs G*G and G*A (see **Note 10**), which are present in “loop E” of the 5S rRNA and the derived scaffold. Initially, the program is trained on the *V. proteolyticus* 5S rRNA sequence. If the predicted secondary structure deviates from the known one, the base pairing parameters should be modified until the prediction yields the correct base pairing pattern. Then, the same set of parameters is used to predict secondary structure of the chimera. If the scaffold part does not fold in the same way as it is seen in the 5S rRNA, the chimera must be redesigned to eliminate erroneous base pairing.

Once the chimeric RNA design has been validated, the appropriate DNAzyme catalytic cores can be selected from the list of well characterized RNA-cutting DNAzymes to suit best the junctions between the cargo RNA and the scaffold (or the spacers, if they are incorporated into the design). For each of the 16 possible types of dinucleotide junction there is at least one known DNAzyme core with substantially high cleavage efficiency [13–17]. Since purine-purine junctions are cleaved easier than any other junction, one may consider adjusting the chimeric RNA design accordingly as long as it is compatible with the cargo RNA sequence and the overall layout of the chimera. The selected catalytic core should be flanked with 10–15 nucleotides-long sequences complementary to the chimeric RNA on both sides of the cleavage site.

3.2 Construction of the RNA Expression Vector and Transformation of the *E. coli* Cells

dsDNA fragment that encodes cargo RNA sequence flanked with spacers and *Bst*EII restriction sites is inserted into pCA2c vector by using the conventional cloning procedure:

1. In a total volume of 50 μ L, 1 μ g ($\sim$ 0.25 pmol) of pCA2c plasmid is digested with 10 units of *Bst*EII restriction enzyme in 1 $\times$ NEBuffer 3. The reaction mixture is incubated at 60 $^{\circ}$ C for 6 h in a 0.2 mL PCR tube with heated lid. The tube is placed on ice for 5 min, then 10 units of calf intestinal alkaline phosphatase is added, and the reaction mixture is incubated at 37 $^{\circ}$ C for additional 2 h.
2. In a total volume of 50 μ L, 100 pmol of dsDNA oligo are digested with 20 units of *Bst*EII restriction enzyme in 1 $\times$

NEBuffer 3. The reaction mixture is incubated at 60 °C for 8 h in a 0.2 mL PCR tube with heated lid.

3. Each reaction mixture is diluted to 100 μ L with water, extracted two times with 100 μ L of neutral phenol, and three times with 200 μ L of chloroform. After last extraction, the aqueous phase is transferred to a fresh 1.5 mL microcentrifuge tube, acidified by adding 10 μ L of SA Buffer, and mixed with 300 μ L of ethanol. The tube is incubated at (–20)°C for at least 1 h. Then, the precipitate is collected by centrifugation at 10,000 $\times g$ and room temperature for 30 min. Supernatant is discarded. The precipitate is washed twice with 70 % ethanol, and air-dried for 15 min at room temperature.
4. Dry pellet of *Bst*EII-treated dsDNA oligo is dissolved in 100 μ L with water.
5. In the tube containing dry pellet of *Bst*EII-digested pCA2c plasmid, the ligation reaction mixture is assembled. The following components are added: 7 μ L of water, 1 μ L of 10 $\times$ T4 DNA ligase buffer, 1 μ L of *Bst*EII-treated dsDNA oligo, and 1 μ L of T4 DNA ligase. The mixture is incubated at 16 °C for 15 h.
6. Electrocompetent *E. coli* JM109 cells (100 μ L) are thawed on ice for 10 min. Then, 1 μ L of the ligation reaction mixture is added to the cells, and the entire volume is transferred to a sterile electroporation cuvette prechilled on ice. The electroporation is performed with 5 ms pulse of 1.5 kV. Immediately after the pulse, 750 μ L of SOC medium is added to the cuvette. Then, the cell suspension is transferred to a sterile 15 mL Falcon tube, and kept on shaker at 37 °C and 250 rpm for 1 h.
7. Aliquots of 5 and 100 μ L are taken out of the cell suspension and spread on ampicillin-containing LB agar plates. The plates are incubated at 37 °C for 15–20 h until the colonies appear.
8. Several (typically eight) individual colonies of average size are selected for the validation of the obtained plasmid construct. Each colony is transferred with sterile toothpick to a 15 mL Falcon tube with 2.4 mL of LB medium containing 0.1 mg/mL ampicillin. The tubes are incubated on shaker at 37 °C and 250 rpm for 14 h (overnight). From each grown bacterial culture, 0.9 mL aliquot is taken out and mixed with 0.45 mL of 30 % glycerol in a sterile 1.5 mL microcentrifuge tube. Thus prepared glycerol stocks are stored at (–80)°C. The rest of the bacterial culture (~1.5 mL) is centrifuged at 10,000 $\times g$ and room temperature for 5 min. Supernatant is discarded, while the cell pellet is used for plasmid purification.
9. Plasmids are purified according to the described RNase-free plasmid purification protocol [18] with modifications. The entire procedure is performed at room temperature. Each cell pellet is resuspended in 150 μ L of TE Buffer. Then, 150 μ L of

Alkaline Lysis Buffer is added, the suspension is mixed by gentle tapping, and incubated for 3 min. The lysate is brought to neutral pH by adding 210 μL of Neutralization Buffer. The solution is mixed by inversion (not vortexed!) and kept on shaker at 200 rpm for 10 min. Cell debris and precipitated material are removed by centrifugation at $10,000\times g$ for 20 min (*see Note 11*). Cleared supernatant is loaded onto a spin column with silica membrane, and centrifuged at $3,000\times g$ for 2 min. The flowthrough is discarded, and the column is washed two times with 400 μL of Salt Wash Buffer, and three times with 400 μL of Ethanol Wash Buffer. All washes are performed at $10,000\times g$ for 1 min, and the eluates are discarded. After the last wash, the spin column is inserted into a fresh 1.5 mL microcentrifuge tube and dried by centrifugation at $10,000\times g$ for 20 min. To elute the plasmid from the spin column, 50 μL of water is applied directly onto the silica membrane. After 5 min of incubation, the eluate is recovered by centrifugation at $10,000\times g$ for 5 min. The collected plasmid solution is stored at $(-20)^{\circ}\text{C}$.

10. Validation of the obtained plasmid constructs is performed by comparing electrophoretic separation profiles of their *Hind*III digests with that of the original pCA2c plasmid (*see Note 12*). Each digestion reaction mixture with total volume of 20 μL contains 0.5 μg of a plasmid and 20 units of *Hind*III restriction endonuclease in $1\times$ NEBuffer 2. The reaction is carried out at 37°C for 2 h, then 4 μL of $6\times$ Agarose Gel Loading Buffer is added, and the entire volume is loaded onto horizontal 2.5 % agarose gel slab with $1\times$ TBE gel buffer containing 0.2 $\mu\text{g}/\text{mL}$ ethidium bromide. Sample with similarly treated pCA2c plasmid is loaded alongside as a control. Hi-Lo dsDNA ladder is used as a DNA size marker (24 μL per well). The electrophoresis is performed at 10 V/cm in the running buffer of the same composition as the gel buffer. When Bromophenol Blue dye reaches the bottom of the gel, the electrophoresis is stopped, and the DNA bands are visualized on a UV transilluminator. *Hind*III digestion of the original pCA2c plasmid produces two bands, 472 and 5,613 bp. The shorter product encompasses the entire *Bst* aRNA gene together with adjacent regions. The presence of the insert in *Bst* aRNA gene is revealed by the decreased electrophoretic mobility of the shorter *Hind*III digestion product. The sequence of the *Bst* aRNA gene bearing the insert may be further confirmed by dideoxynucleotide dye terminator sequencing.
11. The previously prepared glycerol stocks of *E. coli* JM109 carrying the validated derivative of pCA2c plasmid with correct chimeric RNA gene are retained for further use. Those for which the plasmid validation failed are discarded.

3.3 Cultivation and Harvesting of the Transformed *E. coli* cells

The described procedure of *E. coli* cultivation refers to 0.6 L of growth medium.

1. *E. coli* JM109 cells carrying the chimeric RNA expression vector are streaked from glycerol stock on LB agar with ampicillin. The plate is incubated at 37 °C for 15–20 h until the colonies appear.
2. To make starting culture, an individual colony of average size is transferred with sterile toothpick into a 50 mL Falcon tube containing 6 mL of LB medium with 0.1 mg/mL ampicillin. The tube is incubated on shaker at 37 °C and 250 rpm for 14 h (overnight).
3. The entire starting culture is used to inoculate 600 mL of LB medium with 0.1 mg/mL ampicillin in 2.5 L Erlenmeyer flask. The flask is kept on shaker at 37 °C and 250 rpm. Occasionally, an aliquot of 100 µL is taken out and mixed with 900 µL of Culture Diluent for OD₆₀₀ measurement (*see Note 13*). The cultivation is continued until cell density reaches 80 % of the plateau level, which typically takes 6–8 h (*see Note 14*).
4. The bacterial culture is transferred to 250 mL centrifuge tubes (200 mL of culture per tube). The cells are pelleted by centrifugation at 5,000×*g* and 4 °C for 15 min. Supernatant is discarded.
5. Each cell pellet is resuspended in 50 mL of ice-cold TE buffer, and the suspensions are combined in a single 250 mL centrifuge tube of known empty weight. The cells are pelleted by centrifugation at 5,000×*g* and 4 °C for 15 min. The supernatant is discarded, and the tube is weighed to determine the wet cell weight (*see Note 15*). The cell pellet can be either stored at (–80)°C or used immediately for the RNA isolation.

3.4 Cell Lysis and Crude Fractionation of Nucleic Acids

The nucleic acid isolation protocol uses 5 g of wet *E. coli* cells as a starting material. The protocol can be re-scaled if necessary.

1. The cells are thawed on ice for 10–15 min if they were stored frozen, otherwise they can be processed immediately.
2. To 5 g of wet cells in 250 mL centrifuge tube, cold TE Buffer is added to a total volume of 24 mL. Cells are quickly resuspended by vortexing.
3. All following steps are performed at room temperature unless otherwise mentioned. To the cell suspension, 4 mL of TAE-CL Buffer is added. After brief mixing, 4 mL of 10 % SDS and 8 mL of formamide are added, and the suspension is mixed by inversion until the lysis is complete (3–5 min).
4. Cell lysate is thoroughly mixed with 40 mL of Potassium Acetate Buffer, and left on the shaker for 20 min at 200 rpm. At this point, an abundant fibrous precipitate is formed (*see Note 11*).

5. The precipitate is removed by centrifugation at $10,000\times g$ and 20°C for 20 min. Cleared supernatant is transferred to a fresh 250 mL centrifuge tube, where it is mixed with 160 mL of ethanol. The mixture is kept at $(-20)^{\circ}\text{C}$ for at least 1 h.
6. Precipitated nucleic acids are collected by centrifugation at $10,000\times g$ and 4°C for 20 min, and the supernatant is discarded.
7. The precipitate is washed with 100 mL of 70 % ethanol, and dried under vacuum for 30 min (*see Note 16*).
8. Small RNAs are back extracted from the precipitate by shaking it with 40 mL of Sodium Acetate Buffer at room temperature and 250 rpm for 1 h (*see Note 17*). All insolubles are removed by centrifugation at $10,000\times g$ and 20°C for 20 min. Cleared supernatant is transferred to a fresh 250 mL centrifuge tube, and mixed with 100 mL of ethanol. The mixture is kept at $(-20)^{\circ}\text{C}$ for at least 1 h. Precipitated RNA is collected by centrifugation at $10,000\times g$ and 4°C for 20 min, supernatant is discarded.
9. The precipitate is washed three times with 100 mL of 70 % ethanol (*see Note 18*), and dried under vacuum for 30 min (*see Note 16*). At this point, it consists mostly of tRNAs, 5S rRNA, and other cellular RNAs shorter than 500 nt including the chimeric RNA (*see Note 19*). The precipitate can be stored at $(-20)^{\circ}\text{C}$ or used immediately for the chimeric RNA purification.

3.5 Purification of the Chimeric RNA by Preparative Electrophoresis

The protocol refers to 5 mg of starting material that represents low-molecular-weight fraction of *E. coli* RNAs. The RNA species are separated by electrophoresis in 8 % polyacrylamide gel with urea, and visualized by UV shadowing. The band corresponding to the chimeric RNA excised, and the RNA is extracted from the gel by electroelution. All steps are performed at room temperature unless otherwise mentioned.

1. A gel cassette is assembled from square glass plates 20×20 cm and spacers 2 mm thick using paper clips to hold the assembly together.
2. Acrylamide Monomers, 93 mL, are mixed with 6 mL of $10\times$ TBE buffer and 50 μL of TEMED. The polymerization reaction is initiated by adding 950 μL of ammonium persulfate. After that, the gel solution should be poured in the cassette as quickly as possible.
3. The cassette is filled horizontally with the gel solution. Care should be taken not to allow air bubbles to be trapped inside the cassette because around them gel does not polymerize. An one-well comb is inserted, and paper clips are placed along

its whole length to ensure tight contact between glass plates and the comb.

4. The gel is allowed to settle for at least 1 h. Afterwards, the gel cassette can be sealed in a plastic bag and stored refrigerated with the comb in, or used immediately.
5. Prior to mounting the cassette in the gel apparatus, the comb is removed and the well is washed thoroughly with ultrapure water.
6. The cassette is mounted in the gel apparatus, and the electrode chambers are filled with 1× TBE buffer.
7. The gel is pre-run at 600 V until the current is stabilized (*see Note 20*). This may take 1–2 h.
8. The loading sample is prepared by mixing 0.5 mL of aqueous RNA solution (10 mg/mL, *see Note 21*) with 0.5 mL of 2× Polyacrylamide Gel Loading Buffer with Formamide. To unfold RNA, the sample is incubated at 80 °C for 5 min, then kept on ice until loaded.
9. When the gel is ready to be run, the power is turned off, and the well is flushed with nanopure water. The sample is loaded immediately (*see Note 22*).
10. The gel is run at 400 V for 30 min to allow the sample to enter the gel, then the voltage is increased to 600 V. The run continues until xylene cyanol dye reaches the bottom (*see Note 23*).
11. The power is turned off, the cassette is taken out of the gel apparatus and disassembled. The glass plates are removed, the gel slab is transferred onto Saran wrap, and placed over a TLC plate with fluorescent indicator (*see Note 24*). RNA bands are visualized by shining the UV light on the gel from above.
12. The RNA band of interest is excised from the gel with an ethanol-washed razor blade. The excised gel strip is further cut onto smaller pieces to conveniently accommodate them in electroelution unit.
13. Electroelution unit is assembled from 5 mL Falcon tube, 0.2 mL PCR tube, and two pieces of dialysis membrane held in place by silicon rubber rings (Fig. 3). The gel pieces are placed inside the gel chamber, the unit is filled with 0.2× TAE Buffer, and mounted in the apparatus for horizontal electrophoresis filled with the same buffer. Electroelution is performed at 120 V for 8 h. At the end, the electrode polarity is inverted, and the run continues for another 60 s to release RNA from the membrane. Then, all liquid from the gel chamber is carefully pipetted out and discarded. The content of the receptacle (~200 µL) is transferred to a fresh 1.5 mL microcentrifuge tube, acidified with 20 µL of Sodium Acetate Buffer, and mixed with 600 µL of ethanol. The mixture is kept at (–20)°C



Fig. 3 Homemade electroelution unit for quantitative recovery of RNA from polyacrylamide gel. A thin-walled 0.2 mL PCR tube is cut by a razor blade to remove the lid and the bottom. A polypropylene 5 mL Falcon tube is punched near the bottom with red-hot iron nail, and the truncated 0.2 mL PCR tube is immediately inserted into the opening before the molten plastic solidifies. An additional hole is punched over the joint for easy access to the content of the unit. The 5 mL tube serves as a gel chamber while 0.2 mL tube is a receptacle for the eluted RNA. Polyacrylamide gel slice is placed into the gel chamber as close to the joint as possible, and the unit is sealed with two pieces of wet dialysis membrane held in place by silicon rings of appropriate diameter. The unit is filled with buffer, and mounted in an apparatus for horizontal electrophoresis with the receptacle facing the anode, and the gel chamber facing the cathode

for at least 1 h. Precipitated RNA is collected by centrifugation at $10,000\times g$ and $4\text{ }^{\circ}\text{C}$ for 20 min, supernatant is discarded. The pellet is washed twice with 500 μL of 70 % ethanol, air-dried for 15 min, and stored at $(-20)^{\circ}\text{C}$ until needed.

3.6 DNazyme Cleavage of the Chimeric RNA

The procedure applies to the digestion of 10 nmol of the chimeric RNA with a pair of 5'-biotinylated 8–17 DNazymes with E1111 catalytic core each (*see Note 25*).

1. A dry pellet of the chimeric RNA is dissolved in water to a final concentration of 200 μM (*see Note 26*).
2. In a 15 mL Falcon tube, the following reagents are combined in given order: 50 μL of 200 μM chimeric RNA, 790 μL water, 200 μL 1 M MOPS-NaOH (pH 7.2), 500 μL of 200 μM 5'-Cutter DNazyme, 500 μL of 200 μM 3'-Cutter DNazyme, 60 μL 10 mM Spermine-HCl, 500 μL 4 M NaCl, 500 μL 1 M KCl, 300 μL 0.1 MgCl_2 , 600 μL 0.1 M MnCl_2 . The mixture is incubated at $25\text{ }^{\circ}\text{C}$ for 48 h (*see Note 27*).
3. The reaction is stopped by adding 500 μL of Sodium Acetate Buffer followed by 10 mL of ethanol. The solution is mixed by vortexing, and incubated at $(-20)^{\circ}\text{C}$ for at least 1 h. Precipitated nucleic acids are collected by centrifugation at $10,000\times g$ and

20 °C for 20 min, supernatant is discarded. The pellet is washed twice with 1 mL of 70 % ethanol, air-dried for 15 min, and stored at (−20)°C.

**3.7 Separation
of the Excised Cargo
RNA from Other
Components
of the DNAzyme
Cleavage Reaction
Mixture**

The protocol takes advantage of the fact that biotin–streptavidin non-covalent complex remains stable under the conditions when the residual interactions between the DNAzymes and fragments of the chimeric RNA are weakened. This makes it possible to capture biotinylated DNAzymes on streptavidin agarose beads and wash away all RNA pieces including the liberated cargo RNA. Then, the cargo RNA is separated from the remains of the protective scaffold by preparative electrophoresis in polyacrylamide gel. The DNAzymes can be recovered from the beads and reused.

1. Streptavidin Agarose slurry (*see* **Note 28**) is shaken well, and 6 mL of the homogeneous bead suspension is transferred to a 15 mL Falcon tube.
2. The tube is centrifuged in a swinging-bucket rotor at $100\times g$ and room temperature for 1 min to sediment the beads. The liquid is discarded. All following steps utilize the same conditions for bead sedimentation unless otherwise mentioned.
3. The beads are resuspended in 7 mL of TEN Buffer, and sedimented by centrifugation. The supernatant is discarded. The procedure is repeated four times to completely replace storage buffer with the TEN Buffer. Washed beads can be stored at 4 °C until needed as 50 % suspension in TEN Buffer. Immediately prior to use, the beads are sedimented by centrifugation, and all the excessive liquid is removed.
4. A dry mixture of the DNAzymes and the processed chimeric RNA (*see* Subheading **3.6 step 3**) is dissolved in 3 mL of TEN Buffer, and added to 3 mL of wet Streptavidin Agarose beads. The suspension is kept on shaker at 37 °C and 250 rpm for 1 h to allow for the DNAzyme immobilization.
5. The suspension is incubated at 65 °C for 10 min. Then, the beads are sedimented by centrifugation while the tube is still hot (*see* **Note 29**). The supernatant is transferred to a 250 mL centrifuge tube.
6. The beads are resuspended in 7 mL of TEN Buffer, incubated at 65 °C for 10 min, and sedimented by centrifugation while the tube is still hot. The supernatant is taken out and combined with the eluate from the previous step. The procedure is repeated another five times (*see* **Note 30**).
7. The collected eluate (~45 mL) is acidified with 5 mL of Sodium Acetate Buffer, and mixed with 125 mL of ethanol. The mixture is kept at (−20)°C for at least 1 h. The precipitated cargo RNA and scaffold fragments are collected by centrifugation at $10,000\times g$ and 20 °C for 20 min. Supernatant is discarded.

The pellet is washed three times with 1 mL of 70 % ethanol, air-dried for 15 min, and stored at $(-20)^{\circ}\text{C}$.

8. The excised cargo RNA is separated from the remains of the scaffold by preparative electrophoresis performed in the same way as described in Subheading 3.5. Because of the lower load, the gel can be cast 0.75 mm thick.
9. The DNazymes are recovered by repeatedly washing the beads with plain water at elevated temperature. The beads are resuspended in 7 mL of water, incubated at 65°C for 10 min, and sedimented by centrifugation while the tube is still hot. The supernatant is collected after each wash. Usually, 8–10 washes are required to completely elute the DNazymes bound to the affinity matrix. The release of DNazymes from the beads can be monitored by measuring the UV absorbance of the eluate at 260 nm.

4 Notes

1. We have noticed that the efficiency of cytoplasmic accumulation of a given chimeric RNAs varies greatly among different bacterial hosts. The majority of the tested chimeric RNA constructs accumulates well in *E. coli* RecA-deficient strains JM109 and BLR, which are chosen for their better maintenance of recombinant plasmids. However, in several cases the chimeric RNA expression was noticeable only in other *E. coli* hosts (strains N3431 and XL1-Blue). Therefore, it is advisable to test several strains prior to selecting one as an assigned host for the chimeric RNA-expressing vector.
2. pCA2c plasmid vector is a derivative of pKK5-1 [19] in which *E. coli* 5S rRNA gene was replaced with the coding sequence of an artificial RNA derived from *Vibrio proteolyticus* 5S rRNA by removing helix III-loop C segment and introducing *Bst*EII restriction site for easy insertion of cargo sequences [5].
3. Spin columns with the collection tubes are commercially available from several suppliers. We routinely use Qiagen (QIAprep Spin Miniprep Columns, #27115) and Epoch Life Science (EconoSpin All-In-One Silica Membrane Mini Spin Column, #1920-050) with equally good results.
4. We do not use DEPC-treated water to avoid problems caused by the side products of DEPC decomposition.
5. Can be replaced with phenol–chloroform (1:1, v/v) or phenol–chloroform–isoamyl alcohol (25:24:1, v/v/v) mixtures equilibrated with the same buffer. Phenol and its concentrated solutions leave painful burns on the skin, therefore it is recommended to always wear gloves and coat when handling them.

6. Can be replaced with a chloroform–isoamyl alcohol (25:1, v/v) mixture. Expose to chloroform vapor causes dizziness, therefore it is advisable to work with it in a fume hood. Upon prolonged storage, chloroform oxidizes to phosgene that can react with both proteins and nucleic acids. It is recommended to extract chloroform with several changes of 0.1 M sodium bicarbonate solution to completely remove phosgene.
7. We use TBE Buffer of lower concentration than usual. It yields sharper bands on both agarose and polyacrylamide gels albeit at the cost of somewhat slower separation.
8. Both acrylamide and *N,N'*-methylenebisacrylamide are potent neurotoxins and possible carcinogens, and should be handled with utmost care.
9. We observed no significant differences in using DNAzymes with 5'- and 3'-attached biotin. Also, the length of the spacer seemingly does not affect DNAzyme catalytic efficiency or their binding to Streptavidin Agarose.
10. Also, 5S rRNA contains several G*U base pairs. This type of base pairing is usually allowed by default. If not, the program settings should be adjusted to permit them.
11. The precipitate is mostly composed of potassium dodecylsulfate, proteins, and genomic DNA.
12. The presence of the insert in *Bst* aRNA gene might be confirmed by its excision from the plasmid with *Bst*EII restriction endonuclease followed by separation of the reaction products on agarose gel. However, for inserts smaller than 100 bp detection on ethidium bromide-stained gels might be problematic. It was found more convenient to screen the obtained plasmids by cutting them with *Hind*III enzyme, which excises significantly longer fragment containing the entire *Bst* aRNA coding sequence.
13. Typically, *E. coli* JM109 cultures in LB medium can grow to OD₆₀₀ 4–5. Since spectrophotometers cannot measure accurately the absorbance above 1.5, the samples should be diluted prior to measurement.
14. The cells are harvested at the late phase of exponential growth when the degradation rate of cellular RNAs remains relatively low.
15. Under the described conditions, 600 mL of *E. coli* JM109 culture yields 4–6 g of wet cells.
16. Drying time may vary depending on the amount and compactness of the collected precipitate.
17. 16S and 23S rRNAs, which constitute the major mass fraction of the precipitate, are poorly soluble in 3 M Sodium acetate, pH 5. While the amount of unfractionated nucleic acid

extracted from 5 g of wet cells is usually within the range of 100–200 mg, the back extraction procedure allows to reduce it to 10–30 mg of predominantly small RNAs.

18. It is important to remove all salts from the RNA sample prior to fractionating it by electrophoresis in order to avoid band broadening and curving.
19. Besides small RNAs, the precipitate often contains a plasmid that can be seen on electrophoretic profiles.
20. Pre-running the polyacrylamide gel is needed to remove ionized byproducts or unused components of the polymerization reaction.
21. The approximate RNA concentration is calculated from the absorbance at 260 nm using the empirical conversion factor for a structured undenatured RNA for which A_{260} of 1 corresponds to 41 $\mu\text{g}/\text{mL}$ of RNA in protonated form [20]. While some RNAs may not follow this rule, the deviations usually remain within 20 % of the above value. Such an accuracy can be regarded as acceptable for preparing gel loading samples.
22. It is important to load the sample before a substantial amount of the gel buffer and urea diffused into the well. This allows one to obtain compact bands with sharp edges.
23. Xylenecyanol mobility in 8 % denaturing polyacrylamide gel corresponds to approximately 60–65 nt RNA, i.e., it migrates in front of tRNA pool.
24. Instead of the TLC plate, one can use a sheet of common printer paper. Under UV light, it produces fluorescence bright enough for reliable RNA detection on preparative polyacrylamide gels.
25. E1111 catalytic core, TGTCAGCGACACGAA, is best suited for the cleavage of NG junctions, where N is A, U, G, or C [13].
26. Precise molar concentration of the chimeric RNA is determined by hydrolyzing a small amount of it to mononucleotides and measuring the hydrolysate's absorbance at 260 nm. 70 μL of the chimeric RNA solution with A_{260} of 4 is mixed with 30 μL of 1 N NaOH and incubated at 37 °C for 14 h (overnight). The solution is neutralized by 521 μL of untitrated 0.1 M NaH_2PO_4 , and diluted with 379 μL of water to bring the total volume to 1 mL. The A_{260} of the hydrolysate is related to the concentration of the chimeric RNA by the following expression: $A_{260} = \text{CONC} (15,020 N_A + 9,660 N_U + 12,080 N_G + 7,070 N_C)$, where CONC is the RNA concentration in mol/L, and N_A , N_U , N_G , and N_C are the numbers of adenosine, uridine, guanosine, and cytidine nucleotides, respectively, in the chimeric RNA molecule [21].

27. The incubation time may vary considerably depending on the catalytic efficiency of the selected DNAzyme cores, accessibility of the cleavage sites on the chimeric RNA, and the ionic composition of the reaction mixture. Preliminary tests may be needed to optimize the reaction time for a given combination of chimeric RNA and DNAzymes.
28. The protocol refers to Streptavidin Agarose beads with binding capacity of >70 nmol of biotin-tagged ligands per 1 mL of wet beads. If the capacity is lower, the amount of the beads should be increased accordingly.
29. It is recommended to place the thermostat incubator near the centrifuge to minimize the time needed to transfer the tube from the incubator to the centrifuge.
30. In the case of strong residual interactions between the DNAzymes and the fragments of cleaved chimeric RNA, the number of bead washes should be doubled.

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Detection of RNA–Protein Interactions Using Tethered RNA Affinity Capture

Hidekazu Iioka and Ian G. Macara

Abstract

Recent progress in large-scale nucleic acid analysis technology has revealed the presence of vast numbers of RNA species in cells, and extensive processing. To investigate the functions of these transcripts highly efficient methods are needed to analyze their interactions with RNA-binding proteins (RNBPs), and to understand the binding mechanisms. Many methods have been described to identify RNBPs, but none are wholly satisfactory, in part because RNAs are flexible macromolecules that adopt multiple conformations only some of which might bind to specific proteins. Here we describe a novel in vitro RNA-pull-down assay using tRNA scaffolded Streptavidin Aptamer (tRSA), to identify transcript specific RNA binding protein from mammalian cell lysates. The tRNA scaffold functions to stabilize the structure of the aptamer and the attached RNA, increasing the efficiency of the affinity purification.

Key words RNA, Pull down, Aptamer, tRNA scaffold, RBP

1 Introduction

Technological innovation in the high-throughput analysis of nucleic acids has revealed the existence of many classes of noncoding transcripts in addition to thousands of splice variants of mRNAs [1–3]. A substantial fraction of the genome in mammals is transcribed as long noncoding RNAs, mostly of unknown function, and multiple families of small RNAs regulate gene expression. Many mRNAs are modified by posttranscriptional mechanisms such as alternative splicing and RNA editing [4–6]. It is likely that the functions of this universe of RNAs involve sequence- or structure-specific interactions with proteins, and robust and sensitive methods are required, therefore, to interrogate and understand these interactions.

One of the simplest ways to analyze RNA–protein interaction is to attach a functional tag such as GST or His6 to a protein of interest and perform co-precipitations to identify associated RNAs. Conversely, it should be possible to attach a tag to an RNA of interest and pull-down associated proteins. However, RNAs are dynamic

molecules that do not always possess a stable tertiary structure, so effective attachment of a tag to an RNA of interest is not trivial.

RNA aptamers are RNA molecules that possess high affinity for specific molecules, through sequence specific interactions and tertiary structures that are generated by base pairing in molecules of the RNA. Aptamers have been developed empirically against a number of targets by selection from large libraries of random sequences, and they are promising candidates for RNA tags. However, as a consequence of various factors such as the composition of the solution, the presence of nonspecific RNA binding proteins, and degradation, the affinity for the target molecule can be reduced to the point that affinity capture becomes very inefficient. To address these problems, many of the existing aptamer-based RNA pull-down assays require a special matrix, recombinant protein, or multistep biochemical separation [7–9]. An alternative approach is to stabilize the aptamer structure. Ponchon and Dardel demonstrated that endogenous L20 protein could be co-precipitated from bacterial lysate using a tRNA-scaffolded Sephadex aptamer-23SrRNA fusion [10]. We reasoned that this tRNA scaffold technology might provide a great advantage for transcript-specific affinity purification.

By applying tRNA scaffold technology, we established an efficient RNA pull-down method to detect transcript specific RNA binding protein from mammalian cell lysates (*see* Fig. 1) [11]. In this chapter, we describe the detailed procedure for our assay.

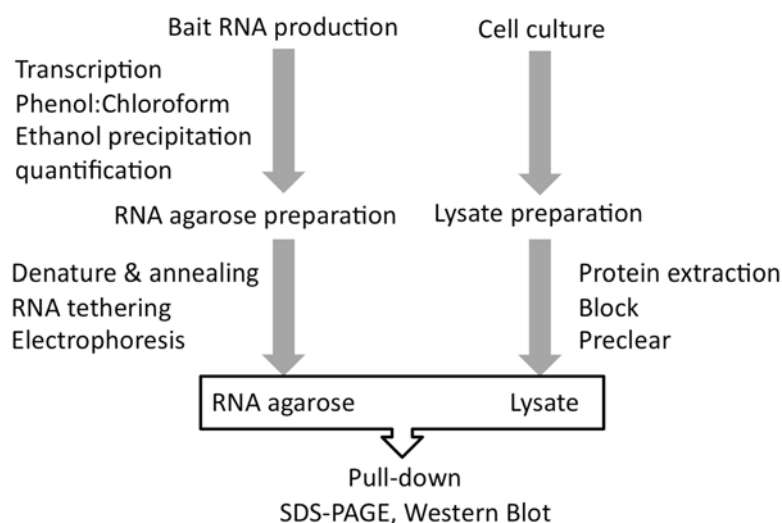


Fig. 1 Timeline for tRNA affinity purification assay. The in vitro synthesis of the bait RNA and attachment to agarose can be performed at the same time as the preparation and preclearing of cell lysates, so that neither has to be stored in the refrigerator prior to use

2 Materials

Particular care must be taken with all reagents to prevent RNase contamination. Basically, all reagents should be treated with DEPC (0.1 %, 1 h~) followed by autoclaving, except for HEPES, DTT, and protein reagents. Molecular Biology grade reagents and weigh boats are usually RNase free, so these reagents only have to be dissolved in DEPC-treated water or RNase-free water. Ideally, reagents for all RNA experiments should be kept on an isolated shelf, and dedicated pipets should be used. In addition, RNA solutions should not be repeatedly frozen and thawed.

2.1 Bait RNA Production

1. Template DNA (Cut plasmid or PCR product) (*see Note 1*).
2. Ampliscribe T7 Flash Transcription Kit (Epicentre).
3. Phenol–chloroform.
4. 3 M NaOAc (pH 5.2).
5. Ethanol.

2.2 RNA Agarose Preparation

1. Streptavidin agarose.
2. Annealing buffer (50 mM HEPES, 50 mM MgCl₂).
3. Lysis Buffer including RNase inhibitor: 10 mM HEPES pH 7.0, 200 mM NaCl, 10 mM MgCl₂, 1 % TritonX-100, 1 mM DTT (Dithiothreitol), protease inhibitors, and RNase inhibitor (Promega). (*see Note 2*).
4. TRIzol (Life technologies).
5. 3 M NaOAc (pH 5.2).
6. Ethanol.

2.3 RNA Electrophoresis

1. 10× MOPS buffer: 0.4 M MOPS (pH 7.0), 0.1 M sodium acetate, 0.01 M EDTA (pH 8.0).
2. Formalin (37 % formaldehyde).
3. Agarose powder.
4. Deionized water.

2.4 Cell Lysate Preparation

1. Lysis Buffer: 10 mM HEPES pH 7.0, 200 mM NaCl, 10 mM MgCl₂, 1 % TritonX-100, 1 mM DTT (Dithiothreitol), and protease inhibitors.
2. White Egg Avidin (EMD chemicals).
3. Yeast RNA (Sigma).
4. Streptavidin agarose.

3 Methods

3.1 Preparation of Bait RNA

1. Follow the manufacturer's protocol for the Ampliscribe T7 Flash Transcription Kit.
2. Add 1 volume of phenol–chloroform to RNA solution and vortex for a few seconds.
3. Centrifuge (4 °C, 16,000×*g*, 5 min).
4. Transfer supernatant to new tube, add 1/10 volume of 3 M NaOAc (pH 5.2) and 2.5 volume of ethanol.
5. Slightly vortex-mix and precipitate RNA at –20 °C for 30 min or more.
6. Centrifuge (4 °C, 16,000×*g*, 10 min).
7. Remove liquid phase and rinse white pellet with 80 % ethanol.
8. Add RNase free H₂O and quantitate RNA concentration by spectrometer.
9. Make aliquots and store at –80 °C.

3.2 Preparation of Cell Lysate

There are various ways to prepare cell lysates. The optimal condition of buffer, salt and detergent must be determined initially to maximize the solubilization of the target fraction. However, because Mg²⁺ plays an important role in the RNA folding, chelating reagents (e.g., EDTA) should not be added to the Lysis buffer [12]. Here we describe the conditions to pull-down endogenous FMRP (*Fragile X Mental Retardation Protein*) from HEK293 cell lysate [13]. Lysate should be kept at 4 °C or on ice throughout the assay.

1. Freshly maintain HEK293 cells on 10 cm dishes.
2. Add 2 mL ice cold lysis buffer and collect cells using cell scraper. Transfer cell lysate to 15 mL conical tube.
3. Homogenize lysate by sonication (Sonics & Material, Vibra-Cell, Duty cycle 50 %, output 4, 10 s×3).
4. Centrifuge lysate (4 °C, 16,000×*g*, 10 min).
5. Transfer supernatant to new tube and quantitate protein concentration by measuring absorbance at 280 nm.
6. To block endogenous biotinylated protein and nonspecific RNA binding proteins, add white egg avidin (10 µg/mg protein), yeast RNA (0.5 mg/mg protein) and incubate at 4 °C for 30 min on rotator.
7. Preclear the lysate by adding 40 µL (bed volume) of streptavidin agarose and incubate at 4 °C for 30 min on rotator.
8. Centrifuge tube (4 °C, 16,000×*g*, 5 min), transfer supernatant to new tube, and add RNase inhibitor (200 U/mL).

3.3 Preparation of RNA Agarose

1. Prepare 30 µg of bait RNA in 1× annealing buffer in 1.5 mL tube (50–100 µL). Bait RNA is heat denatured at 65 °C for 5 min. The tube is then kept at room temperature for 5 min, and finally transferred to ice.
2. Prepare 60 µL streptavidin agarose in 1.5 mL tube, and wash it twice with lysis buffer including RNase inhibitor.
3. Add bait RNA (from Subheading 3.3 step 1) on streptavidin agarose and adjust volume to 300 µL by adding lysis buffer including RNase inhibitor. Incubate tube for 20 min at 4 °C on rotator.
4. Wash Streptavidin Agarose twice with lysis buffer. Half is applied for RNA pull-down, and half is treated with 200 µL Trizol to analyze tethered RNA.
5. Vortex-mix TRIzol added tube and centrifuge (16,000×g, 5 min).
6. Transfer supernatant new tube, and add 1/10 volume of 3 M NaOAc (pH 5.2) and 2.5 volume of ethanol.
7. Vortex briefly and precipitate RNA at –20 °C for 30 min or more.
8. Centrifuge (4 °C, 16,000×g, 10 min).
9. Remove liquid phase and rinse white pellet with 80 % ethanol.
10. Add RNase free H₂O and quantitate RNA concentration by spectrometry.
11. Analyze bait RNA extracted from RNA agarose by formaldehyde agarose gel electrophoresis. (*see Note 3*).

3.4 Pull-Down Assay

1. Add precleared cell lysate on RNA beads. Incubate tube for 1–2 h at 4 °C on rotator.
2. Wash RNA agarose with lysis buffer at least five times.
3. Add sample buffer and separate proteins by SDS-PAGE.

4 Notes

1. In this pull-down assay, tRSA (tRNA scaffolded Streptavidin Aptamer) tagged RNA is employed as a bait RNA. Prior to production of bait RNA in vitro, the template DNA including promoter, tRSA, and RNA of interest must be constructed. We constructed a pcDNA3-tRSA-empty vector (Addgene Plasmid #32200) and positive control vector (pcDNA3-tRSA-G quartet, Addgene #32203), and both of them are available from Addgene. The tRSA tag is cloned in the EcoRI site of pcDNA3 (Invitrogen) (*see Fig. 2*). The RNA of interest should be cloned either upstream or downstream of the tRSA tag.

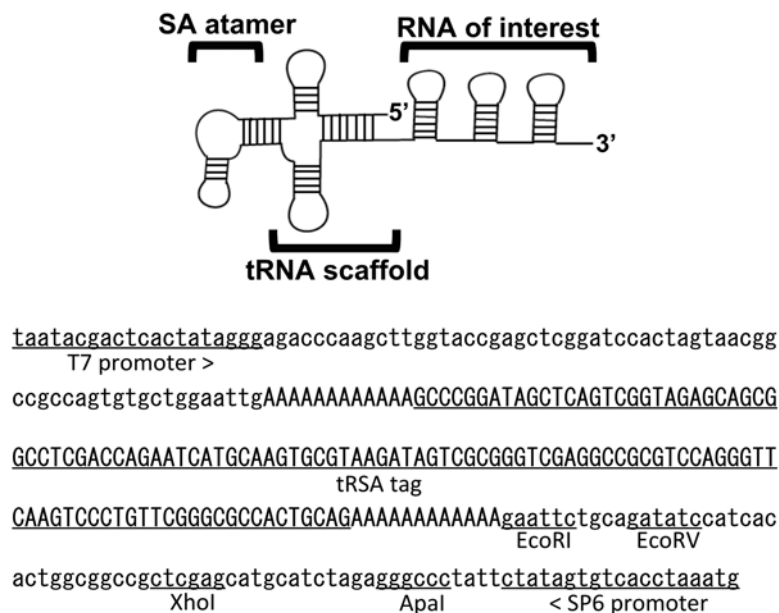


Fig. 2 Structure of the tRSA. *Top* shows a diagram of the predicted secondary structure of the construct, with an RNA of interest attached to the 3' end of the tRNA scaffold. The sequence of the tRSA tag in the vector is shown with useful cloning sites. Expression is driven from the T7 promoter

A PCR product is preferable to employ as a DNA template for in vitro RNA production because we have found that transcription efficiency is usually higher than with cut plasmid. Prepared DNA template should be purified using Qiaquick PCR purification kit (Qiagen).

2. To avoid RNase contamination, premade solutions (5 M NaCl, 1 M MgCl₂, and 10 % TritonX-100) must be treated with 0.1 % DEPC (Diethyl Pyrocarbonate) or prepared with RNase-free water. Because DEPC is reactive to those reagents, 1 M HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and 1 M DTT must be prepared with RNase-free guaranteed reagents and DEPC pretreated water or RNase-free water.
3. Add agarose (1.5 g), 10× MOPS buffer MOPS (10 mL), and 72 mL deionized water to flask, and boil until the agarose is completely dissolved. Keep flask on a magnetic stirrer at room temperature. After the temperature has fallen to 60 °C, add 18 mL formalin (37 % formaldehyde solution) to the flask, mix it thoroughly, and pour formalin gel solution into a mold. This step must be done in a chemical fume hood.

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A Universal Method for Labeling Native RNA in Live Bacterial Cells

Irina Smolina and Natalia Broude

Abstract

The spectrum of RNA functions in the cell continues to widen and new types of RNA molecules continue to be discovered. However, methods to access and manipulate endogenous RNAs in live cells are limited. Here we describe a universal technique for labeling natural RNAs in live cells with the probes synthesized by the cell. The method is based on fluorescent protein complementation in combination with a split aptamer approach. Two RNA probes containing split aptamer sequences flanked with the antisense RNA target sequences are assembled on the target RNA to form a fluorescent ribonucleoprotein (RNP) complex. The mechanism of complex formation ensures highly sensitive RNA detection allowing visualization of endogenous bacterial mRNAs. We demonstrate the great potential of this method by detecting chromosomally low-level expressed unmodified bacterial mRNA in living bacterial cells. This method holds promise to become a broadly used tool in basic research, and eventually in diagnostics and therapeutics.

Key words RNA, Live cells, Split aptamers, Protein complementation, Endogenous bacterial RNA, Fluorescent proteins

1 Introduction

Studies of RNA molecules in their physiological environment within the live cells represent a challenging but indispensable task to fully understand temporal and spatial dynamics of these ubiquitous cellular regulators. RNA labeling in live cells can be achieved using many different approaches (for reviews see [1–5]). These methods can be broadly separated into two groups: the methods that require *prior* RNA modification and those that detect unmodified endogenous RNAs. The vast majority of methods from the first group use genetically engineered probes that exploit internal cellular mechanisms for their synthesis. Therefore, these methods do not require probe delivery. Most of these methods are based on interactions of different aptamers with corresponding RNA-binding proteins that bind cognate aptamers with high affinity. Target RNAs should be genetically engineered to be tagged with

aptamers while the RNA-binding proteins are expressed as fusions with the full-size or split fluorescent proteins [1, 6, 7]. The requirement to tag target RNAs with extra sequences that have strong propensity to form secondary structures poses serious limitations since it is known that aptamers expressed in *cis*- can affect RNA stability, the level of RNA expression and/or translation efficiency [8–10]. Therefore, multiple controls need to be performed to test functionality of the aptamer-tagged RNAs. Notwithstanding great popularity of the most widely used MS2 method belonging to this group, the necessity to tag target RNA limits the usefulness of these methods in diagnostic and therapeutic studies.

The unmodified endogenous RNAs are usually detected by hybridization with the oligonucleotides that change their properties and become fluorescent upon RNA binding [1–5, 7]. Such probes include molecular beacons, auto-ligation probes, and probes making FRET pairs. There are, however, several limitations to these methods as well, such as the sequestration of the probes by the nucleus and the necessity to protect the probes from nucleases. But the major limitation of hybridization probes is that they must be efficiently delivered to the cell.

Here we describe a new technique that combines the flexibility of hybridization-based oligonucleotide probes allowing detection of unmodified RNAs with the live imaging capability of protein complementation approach. The approach is based on RNA-dependent self-assembly of a split aptamer followed by protein complementation, where the RNA probes and fusion proteins are genetically encoded and synthesized by the cell [11] (Fig. 1). Thus the target RNA serves as a scaffold for the formation of a fluorescent ribonucleoprotein (RNP) complex. We demonstrate the great potential of the method by visualizing chromosomally expressed endogenous bacterial RNA. In the course of this study we discovered a mechanism of RNP complex formation suggesting

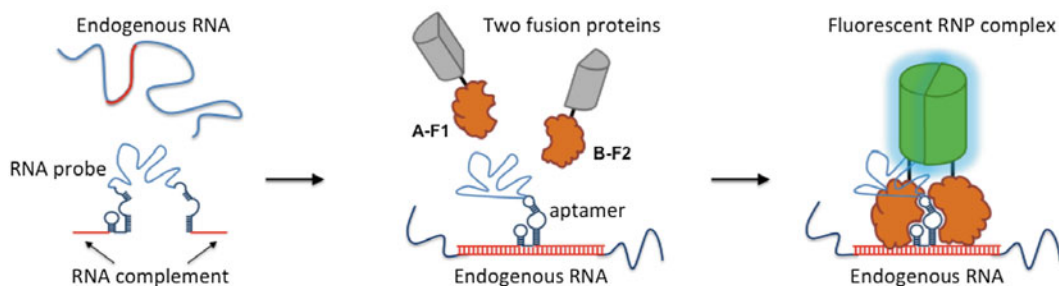


Fig. 1 Schematics of native RNA recognition by the target-specific RNA probes followed by protein complementation. Target RNA hybridizes with RNA probes that have two antisense sequences fused to split aptamer; this triggers the reassembly of the eIF4A-specific aptamer; aptamer folding is followed by the reassembly of the eIF4A protein and EGFP and development of fluorescent signal. A and B are the fragments of EGFP, while F1 and F2 are fragments of the eIF4A protein (from [11])

interactions between the partial aptamer sequences and the split fusion proteins. These interactions result in decreased fluorescent background and facilitate highly sensitive RNA detection.

To the best of our knowledge that is the first example of using split aptamers synthesized by the cell. Split aptamers have been explored extensively in different assays *in vitro*. However, their application *in vivo* has been never tested due to the difficulties of producing short RNA strands with defined ends. For example, split aptamer probes have been used by the Kobatake group to detect short RNA oligonucleotide [12]. In these experiments, both RNA probes and RNA target were delivered to the cell by lipofection. Our work demonstrates that the split RNA probes expressed within the long transcript with the unrelated intervening fragment and the extra flanks are functional and the eIF4A-specific aptamer folds properly in the presence of the target RNA.

2 Materials

2.1 Plasmids Construction

1. dNTP solution (10 mM) containing all four dNTPs.
2. Oligonucleotide primers.
3. Restriction enzymes, thermostable DNA polymerase, and DNA ligase.
4. DNA and RNA purification kits.
5. Agarose and gel electrophoresis equipment.
6. 1× Tris–Acetate–EDTA (TAE) buffer: 40 mM Tris–acetate and 1 mM EDTA, pH 8.3.
7. 10 mg/ml ethidium bromide.

2.2 Bacterial Growth and Induction

1. Competent *E. coli* strains BL21(DE3), *XL-10* (Stratagene), and *DH5aPRO* (Clonetechn).
2. 1 M isopropyl- β -D-thio-galactopyranoside (IPTG).
3. Ampicillin (Amp), 100 mg/ml.
4. Chloramphenicol (Chp) 10 mg/ml in ethanol.
5. Autoclaved Luria–Bertani (LB) media: 10 g/l tryptone, 5 g/l yeast extract, 5 g NaCl, and 1 mM NaOH.
6. Two plasmids, one encoding two protein fusions and another two RNA probes (Fig. 2).
7. Incubators, at 37, 30, and 20 °C.

2.3 Flow Cytometry Analysis

1. Cells expressing fusion proteins and RNA probes induced and un-induced with IPTG.
2. Negative control cells expressing protein fusions and RNA probes directed to a missing target induced and un-induced with IPTG.

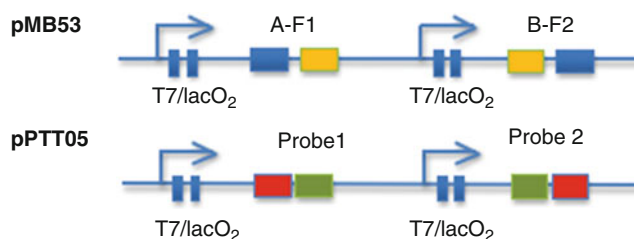


Fig. 2 Two plasmids constructed to label native RNAs in bacterial cells, pMB53 and pPTT05 (for designations of A, B, F1, and F2 see Fig. 1)

3. Cells expressing fusion proteins and RNA probes stressed to induce the RNA target: for example, cells grown under low phosphate stress to induce *PstC* mRNA; or cells under phosphosugar stress to induce *SgrS* RNA.
4. Phosphate-buffered saline (PBS): 150 mM NaCl, 3 mM KCl, 8 mM Na₂HPO₄, and 2 mM KH₂PO₄, pH 7.4.
5. Incubators, at 37 and 25 °C.
6. Flow cytometer with 488-nm excitation filter for collection of green fluorescence.

2.4 Microscopy and Image Analysis

1. Microscope cover slips (22×40×0.15 mm) from Fisher, Pittsburgh, PA, and Lab-Tek two-well glass chamber slides from Nalge Nunc, Naperville, IL.
2. Multi-test microscope slides, 15 wells (e.g., from MP Biomedicals, LLC).
3. The same set of cells as in Subheading 2.3.
4. Filter-sterilized M9 medium: 420 mM Na₂HPO₄, 220 mM KH₂PO₄, 85 mM NaCl, 50 mM NH₄Cl, 1 mM MgSO₄·7H₂O, 0.1 mM CaCl₂·2H₂O, and 0.003 mM Thiamine-HCl.
5. IPTG solution.
6. 0.8 % agarose solution at 50 °C.
7. Inverted fluorescence microscope allowing excitation at 490–500 nm and emission detection at 525–535 nm, equipped with neutral density (ND) filters.

3 Methods

The methods described below outline: (1) preparation of the plasmid expressing target-specific probes with the split aptamer; (2) induction of fusion proteins and RNA probes; and the analysis of bacterial cells by (3) flow cytometry and (4) fluorescence microscopy.

3.1 Expression Plasmids

To visualize endogenous RNA in bacterial cells using fluorescent protein complementation, two compatible plasmids should be used (Fig. 2). The first plasmid (pMB53) expresses two fusion proteins from the two T7/*lacO* promoters in vector pACYCDuet1 (Novagen). This plasmid has been described earlier [11, 13, 14] and is available upon request. The second plasmid is a pETDuet1 derivative and it encodes two RNA probes. Each probe consists of an 11–15 long antisense sequence fused to the fragments of the eIF4A-specific aptamer split at the single stranded loop at position C35/G36 (Fig. 3). Each probe was cloned in one of the two multiple cloning sites (MCS) in plasmid pETDuet1. Because there is no T7 termination sequence between the two MCS the probes are expressed from a first T7 promoter predominantly as a one read-through transcript [11] implying that the two probes are separated by an unrelated sequence (shown as a waved line in Fig. 3).

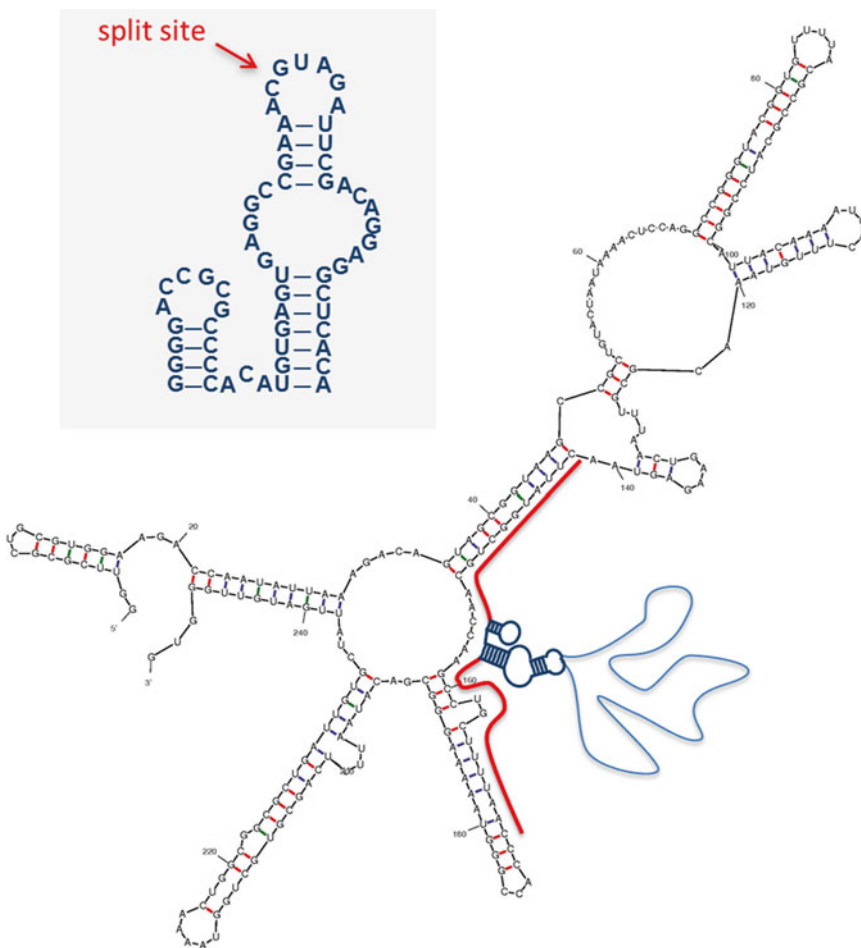


Fig. 3 Schematic representation of the eIF4A aptamer (*inset*) and the aptamer reassembled on the target RNA due to the presence of the antisense arms

Thus, both components of the detection complex are synthesized by the cell and are induced simultaneously by IPTG. If the target RNA is present in the cell, the formation of the RNP complex results in development of the fluorescent signal that reveals RNA presence and localization.

3.1.1 Preparation of the Probes for a New RNA Target

To prepare probe for a new target the antisense sequences in the plasmid pPTT05 (Figs. 2 and 3 marked in red), should be replaced by the sequences complementary to the new target. We suggest using a one-step mutagenesis method described in Unger et al. (15, see **Notes 1** and **2**).

3.2 Induction of Fusion Protein and RNA Probe's

Once the two vectors expressing protein fusions and the RNA probes are available, they have to be transformed into an appropriate bacterial strain expressing T7 RNA polymerase. We used in all experiments *E. coli* strain BL21(DE3). The culture of cells bearing two expression plasmids should be streaked on a plate and a culture started from a single colony. Cells should be grown in LB medium supplemented with 100 mg/ml ampicillin and 34 mg/ml chloramphenicol with vigorous shaking for 3–4 h at 37 °C. For split proteins and RNA probe's expression, cells should be diluted 1:150 in fresh medium supplemented with 0.1–1 mM IPTG (depending on target) and grown overnight with shaking at 25 °C. Target RNA should be induced by the appropriate inducer at the time of IPTG induction. For example, for *PstC* mRNA induction the 3–4 h cell culture was split into two parts; in one part the LB medium was substituted by the low phosphate medium with antibiotics and IPTG (for discussion of negative controls see **Note 3**). The next day, cultures with an OD₆₀₀ between 0.2 and 0.6 should be used for FACS analyses and fluorescent microscopy.

Induction with IPTG initiates the synthesis of both the two fusion proteins and of the RNA probes. By changing IPTG concentration the relative amounts of proteins and probes can be regulated. For the efficient detection of the target RNA the fluorescent background should be very low. The fluorescent background stemming from the spurious self-association of the fusion proteins can be regulated by two means. First, the concentration of the split protein fusions directly depends on IPTG concentration. Second, the mechanism of the RNP complex formation includes an intermediate step, which is the sequestration of the proteins by RNA probes. This step physically separates two fusion proteins and reduces the fluorescent background [11]. The detailed discussion of the problem with the fluorescent background depending on IPTG concentration and the temperature of cell propagation can be found in our publication (16, see **Note 4**).

1. Co-transform bacterial cells, e.g., BL21 (DE3), with the plasmid pMB53 and the plasmid expressing RNA probes,

2. Streak the transformed cultures on LB plates supplemented with 100 mg/ml of ampicillin and 34 mg/ml of chloramphenicol for selection of both plasmids.
3. Pick a single colony from this transformation and start a day culture in 3 ml of LB medium supplemented with 100 mg/ml of ampicillin and 34 mg/ml of chloramphenicol by incubating the cultures at 37 °C for 4 h with vigorous shaking. Concurrently, grow the control cells with the plasmid pMB53 (no RNA probe control) using the same protocol.
4. After 4 h, make multiple inoculations (1, 5, 20, and 50 μ L) in 4 ml of fresh LB media supplemented with antibiotics and 0.1–1 mM IPTG to ensure that a desired optical density (OD_{600}) would be reached overnight and that the densities would be similar between the test and control strains.
5. After overnight growth, split the cell cultures with OD_{600} between 0.2 and 0.4 in half into separate tubes so that RNA synthesis can be induced in one half with the addition of the appropriate inducer (low phosphate for PstC mRNA, α -methylglucoside for SgrS RNA and so on). In some experiments, the induction of the target RNA was performed simultaneously with addition of IPTG.
6. Analyze cell fluorescence by flow cytometry and microscopy, as described below, at different time points after induction (*see* **Note 5**).

3.3 Flow Cytometry Analysis of RNA Labeling

1. Prepare *E. coli* cells as described in Subheading 3.2.
2. Pellet 0.5 ml of cells by centrifugation at 8,000 rpm ($3,600 \times g$). Wash the cells twice with 1 $\times$ PBS. Resuspend the cells in 0.5 ml of PBS.
3. Analyze cell fluorescence using a flow cytometer. Perform excitation with a 488-nm argon laser and use a 515–545 nm emission filter (FL1). Measure fluorescence of 100,000 cells in each sample at a low flow rate. Select a small forward scatter and side scatter gate to decrease fluorescence variation due to different cell sizes. Use WinMDI software to analyze the data.
4. Measure the fluorescence of IPTG-induced cells and non-induced cultures. Also, measure the fluorescence of cells transformed with the plasmid expressing the fusion proteins and RNA probes in absence of target RNA. For this, prepare cells under specific stress and cells in normal conditions (*see* **Note 2**).

3.4 Time-Lapse Analysis of Cell Fluorescence and RNA Localization by Microscopy

1. Prepare a 0.8 % agarose solution in 1 $\times$ PBS. Allow the solution to equilibrate to 50 °C and keep it warm at 50 °C prior to use.
2. Distribute small droplets of agarose into 15 wells of a multi-test slide. Do not make the agarose pads too big as they will prop open the cover slip.

3. Immediately dispense an aliquot of cell culture over the wells such that it is enough to cover them. Allow the cells to settle on the pads for 4 min at room temperature.
4. Carefully aspirate excess liquid covering the pads using a vacuum aspirator. Take care not to disrupt or remove the pads from the slide.
5. Cover the slide with a long cover slip, e.g., 24 × 50 mm.
6. Image the cells in time-lapse format using an inverted fluorescence microscope with a 100× oil immersion objective and epi-fluorescence system that allows for excitation at 490–500 nm and emission detection at 525–535 nm (exposure time 100–400 ms and 5–10-min intervals). Take differential interference contrast images also to record changes in cell morphology and cell division.
7. Analyze total cell fluorescence and fluorescence distribution within the cell using public accessible (ImageJ) or custom software. Also, compare these images with those obtained with the control cells expressing fusion proteins in absence of RNA (Fig. 4).

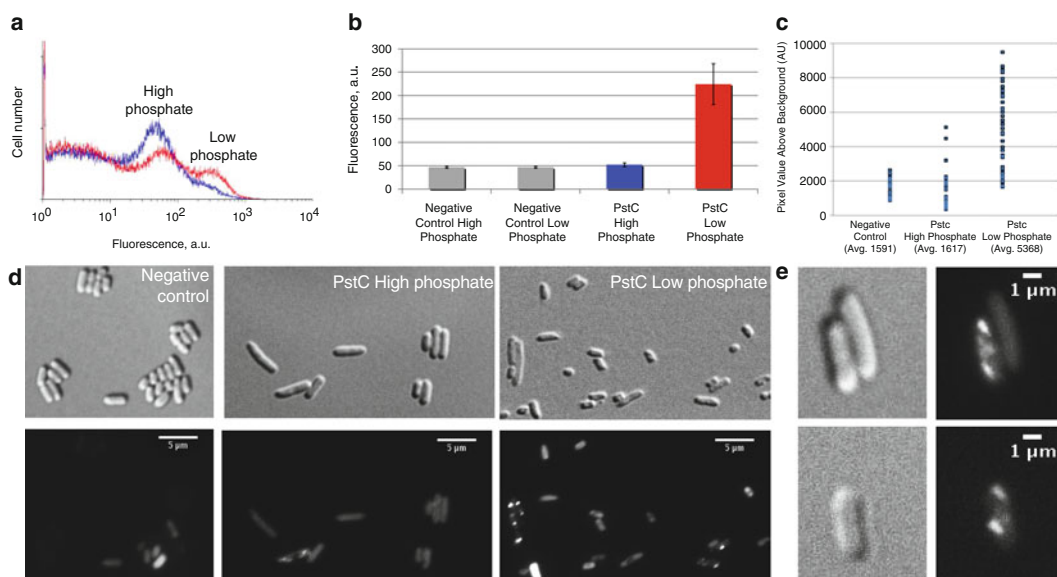


Fig. 4 Fluorescent analyses of *E. coli* cells with chromosomally expressed *PstC* mRNA labeled using protein complementation and split aptamer approach. **(a)** A representative experiment on FACS analyses of the *E. coli* cells with labeled *PstC* mRNA grown in high (blue) and low (red) phosphate; **(b)** Histograms obtained from the FACS data. Average of three independent experiments $\pm$ SD is shown; **(c)** Total cell fluorescence obtained from the fluorescent images of single cells using fluorescent microscopy; **(d)** Fluorescent patterns of labeled *PstC* mRNA in cells in high and low phosphate media. The *left panels* show cells expressing fusion proteins and the probes antisense to β -globin mRNA (negative control). Scale bar, 5 μ m; **(e)** Comparison of *PstC* mRNA patterns in live (*top*) and fixed cells (FISH results, *bottom*). Scale bar, 1 μ m

4 Notes

1. The important step in this method is the choice of the accessible site in target RNA. In this study we used either Mfold (*PstC* mRNA, *SgrS* RNA) or the site known from the previous studies to be accessible (b-globin mRNA). To increase the probability of success it is advisable to prepare two probes targeting two different sites. To detect low-concentration mRNAs (*PstC* mRNA) we used 30 nt long sequences localized in single-stranded loops or in single-stranded loops and the adjacent low-stability ds regions.
2. The plasmids expressing two fusion proteins and the two RNA probes are available upon request from our laboratory. The plasmid pMB53 expressing fusion proteins A-F1 and B-F2 has been described earlier [13, 14]. This is a derivative of pACYC-Duet1 with the chloramphenicol resistance. The two RNA probes have been cloned in the two MCSs of the plasmid pET-Duet1 with ampicillin resistance gene. Our experiments on different design of RNA probes showed that the two RNA probes expressed within one long transcript display lower background fluorescence as compared with the case when the two probes are expressed as two separate transcripts. The detailed study of the mechanism of the RNP complex formation suggested an intermediate step when the fusion proteins bind two fragments of the split aptamer and prevent their spontaneous reassociation [11]. Therefore, we advise to use the same design for all probes. Any plasmid with the two probes for a new RNA target can be obtained from the plasmid pTT05 by a simple one-step mutagenesis to substitute one set of the antisense probes for other.
3. The negative controls are as usually extremely important. If the target RNA is conditionally expressed, the controls should include the cells grown in two conditions: with and without RNA. If the target RNA is constitutively expressed, we recommend using the probes with the corrupted antisense sequences.
4. Efficiency and sensitivity of RNA detection. Since all components of the fluorescent RNP complex are internal and are synthesized by the cell it is important to understand the effect of their relative concentrations on efficiency and sensitivity of RNA detection. From one hand, due to the interactions between the RNA probes and fusion proteins (*see Note 2*), the concentrations of the RNA probes should exceed concentrations of the proteins. Indeed, the excess of RNA probes should result in efficient binding of split proteins and will reduce the background. From the other hand, the large excess of the RNA probes over the proteins will reduce the proportion of the

probes with the two binding sites occupied by the proteins and this will decrease the efficiency of RNA detection.

In our experiments induction with 1 mM IPTG resulted in the concentration of the RNA probes about twofold over concentration of the fusion proteins: 10,000 RNA probe molecules versus 5,000 molecules of the fusion proteins per cell [11]. This ratio provided 75 % reduction of the background fluorescence; however only 25 % of the probes at this ratio have both binding sites occupied by the proteins due to statistical probability. As a result, only these 25 % probes are efficient in detecting the target. Additionally, due to the non-synchronized bacterial cell population different cells have fusion proteins on different stages of expression and protein maturation making some cells non capable of developing fluorescence. All these factors explain that only 17–20 % of cells displayed signal with b-globin mRNA, even though the average RNA concentration was about 75 mol of target RNA [11].

Our results demonstrated very high sensitivity of the method. In live cells we detected signal from *PstC* mRNA with the average RNA concentration ≤ 1 mol/cell which implies that the method has sensitivity close to a single molecule. The bleaching experiments revealed cells with one-to-three step-wise fluorescent decrease traces. We explain this high sensitivity by the combination of three factors. First, protein complementation reduces fluorescent background 10–100-fold as compared to the full-size FPs [13, 14, 16]. Second, the mechanism of split protein sequestration by the RNA probes prevents split protein reassociation and their ability to become fluorescent in absence of the target RNA. This adds an additional 4–5-fold background reduction and allows visualizing signals from single molecules. Finally, *PstC* mRNA happens to be localized; therefore, its fluorescence signal is not spread to the entire cell by diffusion during the image acquisition time and is not overwhelmed by cellular autofluorescence.

5. Detection of the fluorescent signal can be performed using two methods: FACS analyses and fluorescent microscopy. Ideally both methods will produce consistent results. However, if the concentration of RNA is very low, that is often the case with endogenous RNAs, it is also possible that the measurements of the bulk cell culture will not reveal the signal. In this case, fluorescent microscopy can be more sensitive, especially, if RNA is localized. The RNP complex assembled on target RNA includes a short stretch of dsRNA, which can affect RNA stability. To address this question we performed RT-PCR on RNAs isolated from the cells with the RNP complex and the control cells without the complex. We did not find any difference in steady state concentrations of the target RNA which suggests that stability of RNA is not affected by the complex formation.

Acknowledgements

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Live Cell Imaging Using Riboswitch-Spinach tRNA Fusions as Metabolite-Sensing Fluorescent Biosensors

Colleen A. Kellenberger, Zachary F. Hallberg, and Ming C. Hammond

Abstract

The development of fluorescent biosensors is motivated by the desire to monitor cellular metabolite levels in real time. Most genetically encodable fluorescent biosensors are based on receptor proteins fused to fluorescent protein domains. More recently, small molecule-binding riboswitches have been adapted for use as fluorescent biosensors through fusion to the in vitro selected Spinach aptamer, which binds a pro-fluorescent, cell-permeable small molecule mimic of the GFP chromophore, DFHBI. Here we describe methods to prepare and analyze riboswitch-Spinach tRNA fusions for ligand-dependent activation of fluorescence in vivo. Example procedures describe the use of the Vc2-Spinach tRNA biosensor to monitor perturbations in cellular levels of cyclic di-GMP using either fluorescence microscopy or flow cytometry. The relative ease of cloning and imaging of these biosensors, as well as their modular nature, should make this method appealing to other researchers interested in utilizing riboswitch-based biosensors for metabolite sensing.

Key words Fluorescence imaging, Fluorescence microscopy, Flow cytometry, RNA biosensor, Cyclic dinucleotide, Cyclic di-GMP, Vc2 riboswitch, Spinach aptamer

1 Introduction

Fluorescent biosensors provide a measureable output that changes in real time in response to binding of a specific small molecule, making them ideal tools for quantifying and tracking cellular signals in vivo. Most biosensors employed in live cell imaging utilize Förster resonance energy transfer (FRET) between two fluorescent proteins fused to a receptor protein [1]. However, many small molecule metabolites and signals exist for which no corresponding protein receptor is known. Furthermore, engineering a biosensor from a natural protein receptor that produces a large change in FRET signal remains difficult.

Riboswitches are natural RNA-based receptors for metabolites and signaling molecules that bind cognate ligands with high selectivity and affinity, making them strong candidates as biosensor scaffolds [2]. Previously, reporter systems have been developed

using riboswitches to control the expression of a downstream reporter gene such as β -galactosidase or GFP [3]. These systems have the advantage of providing signal amplification, but the read-out of metabolite concentrations is indirect and not responsive in real-time.

The recent development of the Spinach aptamer [4] has enabled the generation of RNA-based fluorescent biosensors that function in vivo. Spinach is an in vitro selected RNA aptamer that binds to 3,5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI), a pro-fluorescent small molecule analog of the GFP chromophore. The Spinach aptamer forms a G-quadruplex motif structure that binds DFHBI and shields the chromophore from solvent- and rotation-mediated forms of energetic decay, causing a 1,000-fold increase in fluorescence [5, 6]. Furthermore, it was shown that DFHBI binding and fluorescence activation by Spinach can be modulated by controlling the formation of the P2 stem with a small molecule-binding aptamer fused to this stem [7]. Following this strategy, we have used a natural riboswitch aptamer to develop a fluorescent biosensor for cyclic di-GMP [8, 9], which is a key second messenger in bacteria that regulates processes including biofilm formation, cell cycle progression, and virulence [10].

Here we describe the application of an RNA-based biosensor for live cell imaging of the bacterial second messenger cyclic di-GMP. We demonstrate two methods, fluorescence microscopy and flow cytometry, to analyze changes in cyclic di-GMP levels in *Escherichia coli* upon co-expression of the biosensor and diguanylate cyclase enzymes. These procedures are expected to be generalizable for other Spinach-based biosensors developed for different cellular metabolites.

2 Materials

2.1 Equipment and Supplies

1. Micropipettor.
2. Vortex mixer.
3. Microcentrifuge.
4. Millipore water filter with a BioPak unit.
5. Micropipettor tips.
6. 1.5 mL microcentrifuge tubes.
7. Sterile filter units.
8. PCR thermocycler.
9. 14 mL Culture tubes and petri dishes.
10. Incubator shaker set to 37 °C.
11. VWR VistaVision Cover Glasses No. 1 ½.
12. VWR VistaVision Microscope Slides.

13. Zeiss 200M AxioVert microscope (Zeiss, Jena, Germany) equipped with a mercury light source X-Cite 120 Series (Exfo Life Science Divisions, Ontario, Canada), a 63×/1.4 Plan-Apochromat oil DIC objective lens and a 1.6× tube lens. For monitoring fluorescence, a GFP filter set with an excitation 470/40 BP, FT 495 beamsplitter, and emission 525/50 BP was used (Filter Set 38 HE).
14. ImageJ data analysis software.
15. 5 mL Falcon polypropylene tubes with cell-strainer cap.
16. BD Influx v7 cell sorter with BD FACS Software (Version 1.0.0.650).

2.2 Reagents

1. Phusion DNA polymerase with 5× HF and 5× GC buffers (NEB).
2. 10× dNTPs (2 mM each ATP, CTP, GTP, TTP).
3. 1× TAE buffer: 40 mM Tris-HCl, 20 mM acetic acid, 1 mM EDTA, pH 8.4.
4. 1 % agarose solution: 1 g agarose in 100 mL 1× TAE buffer.
5. 2-log DNA ladder (NEB).
6. Gel extraction or PCR cleanup kits.
7. Restriction enzymes: BglII & XhoI with NEBuffer 3.1 or EagI-HF & SacII with NEB CutSmart buffer along with NdeI & XhoI with NEB CutSmart buffer.
8. Calf intestinal alkaline phosphatase (Fisher).
9. T4 DNA ligase with 10× ligase buffer (NEB).
10. Luria Broth (LB, Fisher Scientific).
11. *E. coli* TOP10 chemically competent cells (Life Technologies).
12. LB/agar.
13. Carbenicillin: 50 mg/mL stock concentration, filtered through a 0.2 µm nitrocellulose filter.
14. Kanamycin: 50 mg/mL stock concentration, filtered through a 0.2 µm nitrocellulose filter.
15. pET31b(+) plasmid.
16. pET24a or pCOLADuet-1 plasmid.
17. 40 µM stock concentration of the following DNA oligonucleotide primers (**BOLD** = T7 promoter; UNDERLINED = tRNA scaffold; CAPS = Spinach sequence; **BOLD UNDERLINED** = T7 terminator; *ITALICS* = restriction enzyme recognition site; lower case = other):
 - (a) 5'-cgatcccgcgaaat**TAATACGACTCACTATAGGG**
GCCCGGATAGCTCAG TCGGTAGAGCAGCGGCCG
GACGCGACTGAATGAAATGGTGAAGGACGGG.

- (b) 5'-AGAGGCCCAAGGGGTTATGCTATGGCG
CCCGAACAGGGACTTG AACCCTGGACCCGCGG
CCGGACGCGACTAGTTACGGAGCTCACAC
TCTACTC.
- (c) 5'-cagtcaAGATCTcgatcccgcaaataAATACGACTCAC
TATAGGG.
- (d) 5'-catcagCTCGAGCAAAAAACCCCTCAAGA
CCCGTTTAGAGGCCCA AGGGGTTATGCTA.
- (e) 5'-gatcCGGCCGGACGCGACTGAATGAAATGGTG.
- (f) 5'-catgCCGCGGCCGGACGCGACTAGTTACGG
AGCTC.
- (g) 5'-cttgCATATGcacaaccctcatgagagcaag.
- (h) 5'-catgCTCGAGtcagcccgccggggc.
18. *E. coli* BL21 (DE3) Star chemically competent cells (Life Technologies).
 19. Borate buffer: 2.1 g/L boric acid, 9 g/L sodium tetraborate decahydrate; pH to 8.5. Filter through a 0.2 µm nitrocellulose filter and store at ambient temperature.
 20. 1 M HCl solution.
 21. 24× Poly-D-Lysine solution: 2 mg/mL poly-D-Lysine hydrobromide (Sigma, CAS: 27964-9904). Filter through a 0.2 µm nitrocellulose filter and store at -20 °C.
 22. Ethanol.
 23. M9 minimal media, pH 7.0: 48 mM Na₂HPO₄, 22 mM KH₂PO₄, 8.6 mM NaCl, 20 mM NH₄Cl, 5 mM MgSO₄, 0.4 % glucose, 100 µM CaCl₂.
 24. DFHBI (3,5-difluoro-4-hydroxybenzylidene imidazolinone): stock concentration of 20 mM DFHBI in DMSO, stored in 50 µL aliquots at 20 °C.
 25. 1× PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4.

3 Method

3.1 Construction and Transformation of Biosensor and Enzyme Constructs

In these experiments, two separate plasmids are used to separately express the biosensor or the enzyme (*see Note 1*). The Vc2-Spinach tRNA biosensor is cloned into pET31b and alleles of the WspR diguanylate cyclase from *Pseudomonas fluorescens* [11] are cloned into pET24a or pCOLADuet-1. Previous reports have shown that plasmids containing the same origin of replication (e.g., pET vectors) and different antibiotic resistances are incompatible for co-expression as both are not stably maintained in the cell due to competition for the same replication factors [12]. On the contrary, recent reports have shown that the degree of incompatibility

depends on the expression system and that plasmids with the same origin can stably persist for several overnight growth cycles [13]. We have found that both incompatible plasmids (pET31b and pET24a) as well as compatible plasmids (pET31b and pCOLA-Duet-1) function in these experiments to detect altered levels of cdiG, but it is advised to use compatible expression vectors for highly sensitive experiments in order to minimize plasmid loss during bacterial growth.

Though the expression of both the biosensor and enzyme are driven by IPTG-induction in the following experiments, use of the RNA-based biosensor is highly adaptable for many experimental systems. For instance, various promoters can be cloned directly upstream of the Vc2-Spinach tRNA construct to achieve either constitutive or inducible biosensor expression in *E. coli* or other bacterial species. Various enzyme alleles, gene homologs, or mutant enzyme libraries can be coexpressed to assess in vivo enzymatic activity. Furthermore, the biosensor can be expressed alone to study cyclic dinucleotide levels in different strains or in bacterial transposon libraries.

3.1.1 Cloning of Biosensor Expression Vector

For stable expression in vivo, the riboswitch-Spinach fusion, WHICH IS the construct used for in vitro experiments, is inserted within a tRNA^{Lys3} scaffold to reduce susceptibility to RNases and to ensure homogenous end processing in cells (Fig. 1a) [14]. In vitro analysis has confirmed that placement of the Vc2-Spinach construct within the tRNA scaffold has little to no effect on the ligand binding affinity [8]. For initiation and termination of transcription, respectively, the Vc2-Spinach tRNA construct is flanked

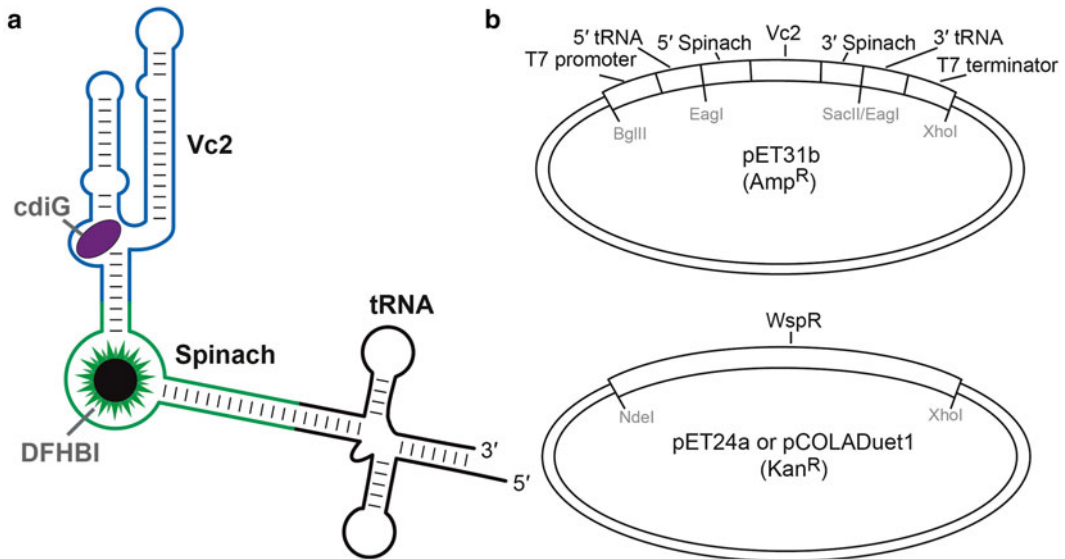


Fig. 1 Design of RNA-based fluorescent biosensor and enzyme constructs. **(a)** Model of Vc2-Spinach tRNA construct bound to cdiG and DFHBI. The tRNA scaffold serves to stabilize the biosensor fold in vivo. **(b)** Diagram of biosensor and enzyme expression plasmid constructs

by T7 promoter and T7 terminator sequences. The existing T7 promoter sequence in the expression plasmid pET31b is removed during the restriction digest to ensure homogenous expression of the RNA from a single promoter site.

Two procedures for constructing the biosensor expression plasmid are described below. In the first case, the Vc2-Spinach tRNA insert is generated through PCR and cloned into the original pET31b vector (*see Note 2*). Alternatively, the Vc2-Spinach biosensor can be cloned into a pET31b plasmid already containing the tRNA scaffold (Fig. 1b). The second strategy is recommended when the user already has a biosensor expression plasmid and is interested in swapping out the specific biosensor construct for another. Shown below is the sequence of the Vc2-Spinach tRNA biosensor construct (*italics*=restriction enzyme recognition site; **BOLD**=T7 promoter; UNDERLINED=tRNA scaffold; CAPS=Spinach sequence (*see Note 3*); lowercase=Vc2 sequence; BOLD ITALICS=T7 terminator):

*agatct***CGATCCCGCGAAATTAATACGACTCACTA**
TAGGGGCCCGGATAGCTCAGTCGGTAGAGCAGCG
GCCGGACGCGACTGAATGAAATGGTGAAGGACGGGTCC
Acacgcacagggcaaaccattcgaaagagtgggacgcaaagcctccggcctaaaccagaa
gacatggttaggtagcggggttaccgatgTTGTTGAGTAGAGTGTGAGCT
CCGTAACTAGTCGCGTCCGGCCGCGGGTCCAGGGTTCA
AGTCCCTGTTTCGGGCGCCATAGCATAACCCCTTGGGGC
CTCTAAACGGGTCTTGAGGGGTTTTTTGctcgag

Procedure Starting
from Original
pET31b Vector

Step 1a: Generation of Vc2-Spinach tRNA construct (Estimated time: 2 h).

1. To prepare the tRNA-scaffold biosensor insert, combine the following in a PCR tube:
 10 µL 5× Phusion HF buffer.
 5 µL 10× dNTPs.
 0.8 µL 40 µM primer a.
 0.8 µL 40 µM primer b.
 1 µL Vc2-Spinach DNA template (10–50 ng) (*see Note 4*).
 31.4 µL ddH₂O.
 1 µL Phusion DNA polymerase.
2. Amplify the construct using the following standard thermocycler protocol: initial denaturation 98 °C for 1 min; 35 cycles of 98 °C for 5–10 s, 66 °C for 20 s, 72 °C for 15 s, final extension 72 °C for 5 min.
3. Analyze the product on a 1 % agarose gel (expected size: 290 bp). Purify the product via commercial gel extraction or PCR cleanup kits following the manufacturer's protocol and elute the product in ddH₂O or the provided elution buffer.

Step 2a: Generation of Vc2-Spinach tRNA construct with T7 promoter and restriction sites (Estimated time: 2 h).

1. Complete construction of the biosensor construct containing restriction enzyme recognition sites by combining the following in a PCR tube:
10 μ L 5 $\times$ Phusion HF buffer.
5 μ L 10 $\times$ dNTPs.
0.8 μ L 40 μ M primer c.
0.8 μ L 40 μ M primer d.
1 μ L Vc2-Spinach-tRNA DNA template (10–50 ng).
31.4 μ L ddH₂O.
1 μ L Phusion DNA polymerase.
2. Amplify the construct using the same protocol as in **step 1.2** above, but with an annealing temperature of 68 °C.
3. Analyze and purify the product (expected size: 338 bp) as in **step 1.3** above.

Step 3a: Cloning of biosensor insert into expression vector (Estimated time: 2 days)

1. To insert the Vc2-Spinach tRNA construct into the expression plasmid pET31b, perform a double digest on both the insert and the vector using BglII and XhoI restriction enzymes. Prepare 50 μ L digestion reactions by combining either 43 μ L of the complete Vc2-Spinach-tRNA construct or pET31b plasmid with 5 μ L NEBuffer 3.1, 1 μ L BglII, and 1 μ L XhoI.
2. Incubate the reaction at 37 °C for 2 h.
3. To reduce plasmid re-ligation, dephosphorylate the digested pET31b vector by adding 1 μ L Calf Intestinal Alkaline Phosphatase to the digested reaction and incubate at 55 °C for 30 min.
4. Check the digested and dephosphorylated products on a 1 % agarose gel to ensure the integrity of the products and to check that the plasmid was linearized. Purify the products via commercial PCR cleanup kit following the manufacturer's protocol and elute the DNA in ddH₂O or the provided elution buffer.
5. Ligate the digested products by combining the following in a PCR tube: 1 μ L 10 $\times$ T4 DNA ligase buffer, 25 ng digested/dephosphorylated plasmid, 20 ng digested insert, ddH₂O to 9.5 μ L, and 0.5 μ L T4 DNA ligase. Incubate the reaction for 10 min at room temperature or at 16 °C overnight.
6. Deactivate the ligase by heating it to 65 °C for 10 min.

7. Use 1–5 μL of the ligation reaction to transform 50 μL *E. coli* TOP10 chemically competent cells following the manufacturer's protocol. Plate the cells on LB/Carb (50 $\mu\text{g}/\text{mL}$ Carb) plates and incubate 12–16 h at 37 °C.
8. Check transformed colonies for clones with the correct sequence by streaking single colonies on a LB/Carb master plate. Inoculate overnight LB/Carb cultures from the master plate, then isolate the plasmids using a commercial kit and submit the plasmids for sequencing. Use only a plasmid containing the desired sequence for future experiments.

Alternative Procedure
Starting from Existing
Biosensor Construct

Step 1b: Generation of biosensor-Spinach insert

1. Amplify the Vc2-Spinach insert with EagI and SacII restriction sites by combining the following in a PCR tube:
10 μL 5 $\times$ Phusion HF buffer.
5 μL 10 $\times$ dNTPs.
0.8 μL 40 μM primer e.
0.8 μL 40 μM primer f.
1 μL Vc2-Spinach-tRNA DNA template (10–50 ng).
31.4 μL ddH₂O.
1 μL Phusion DNA polymerase.
2. Amplify the construct using the following thermocycler protocol: Initial denaturation 98 °C for 1 min; 35 cycles of 98 °C 5–10 s, 66 °C for 20 s, 72 °C for 15 s, final extension 72 °C for 5 min.
3. Check the product on a 1 % agarose gel (expected size: 175 bp) and purify the product via commercial PCR cleanup or gel extraction kits following the manufacturer's protocol and elute the DNA in ddH₂O or the provided elution buffer.

Step 2b: Cloning of biosensor insert into expression vector (Estimated time: 1 day)

1. Digest the pET31b-Spinach-containing plasmid and the Vc2-Spinach insert by combining either 43 μL of the Vc2-Spinach construct or pET31b-Spinach plasmid with 5 μL NEB CutSmart buffer, and 1 μL SacII. Incubate the reaction at 37 °C for 1 h, then add 1 μL EagI-HF and digest at 37 °C for another hour. It is necessary to do a sequential digest since first cutting with SacII eliminates the second EagI restriction enzyme recognition site.
2. Follow **steps 3a.3–3a.8** above to ligate, transform, and sequence to confirm the pET31b Vc2-Spinach-tRNA biosensor construct.

3.1.2 Construction of Enzyme Expression Vector

Step 4: Generation of WspR diguanylate cyclase insert (Estimated time: 3 h)

1. Amplify the WspR diguanylate cyclase insert by combining the following in a PCR tube:
10 μ L 5 $\times$ Phusion GC buffer.
5 μ L 10 $\times$ dNTPs.
0.8 μ L 40 μ M primer g.
0.8 μ L 40 μ M primer h.
1 μ L WspR DNA template (10–50 ng).
3 μ L DMSO.
28.4 μ L ddH₂O.
1 μ L Phusion DNA polymerase.
2. Amplify the construct using the following thermocycler protocol: Initial denaturation 98 °C for 1 min; 35 cycles of 98 °C 5–10 s, 68 °C for 20 s, 72 °C for 45 s, final extension 72 °C for 10 min (*see Note 5*).
3. Check the amplified product on a 1 % agarose gel (expected size: 1,061 bp) and purify the product by commercial PCR cleanup or gel extraction kits following the manufacturer's protocol and elute the DNA in ddH₂O or the provided elution buffer.

Step 5: Cloning of WspR diguanylate cyclase into expression vector (Estimated time: 2 days)

1. Digest the amplified WspR insert along with pET24a or pCOLADuet-1 plasmid by combining the following: 43 μ L of either the WspR insert or the plasmid with 5 μ L NEB CutSmart buffer, 1 μ L NdeI, and 1 μ L XhoI. Incubate the reaction at 37 °C for 2 h.
2. Follow **steps 3a.3–3a.8** in Subheading 3.1.1, but with cultures grown in LB/Kan (50 μ g/mL) in order to obtain sequence-confirmed pET24a or pCOLADuet-1 WspR plasmid.

3.1.3 Transformation of Expression Vectors for Live Cell Imaging

Step 6: Generation of strains containing both biosensor and enzyme constructs (Estimated time: 1 day)

1. To prepare cells co-expressing the biosensor and enzyme, transform BL21 (DE3) Star *E. coli* cells with ~60 ng each of the pET31b and pET24a constructs following the manufacturer's protocol.
2. Plate the cells on LB/Carb/Kan (Carb: 50 μ g/mL, Kan: 50 μ g/mL) plates and incubate at 37 °C for 12–16 h. Colonies should contain both plasmids and the plates can be stored at 4 °C for ~3 weeks.

3.2 Live Cell Imaging of the RNA-Based Biosensor

In general, cellular imaging with Spinach-based biosensors requires that several conditions be met, including that DFHBI can diffuse into the cell and that the RNA biosensor is expressed at sufficient levels for visualization. The permeability of DFHBI depends upon the composition of the cell membrane or cell wall and thus may fluctuate between different bacterial species and strains or under different growth conditions. Similarly, RNA expression levels may vary between different conditions or stressors on the cell (e.g., co-expression of a heterologous enzyme). Thus, to ensure that observed fluorescence changes are due to changes in metabolite or signaling molecule levels rather than changes in intracellular DFHBI and RNA concentrations, it is recommended that the Spinach aptamer alone be used as a control. If cellular fluorescence of the Spinach-tRNA construct with DFHBI and RNA expression levels remain constant under the experimental conditions, then differences in biosensor fluorescence under these conditions should provide an accurate readout of changing metabolite levels.

Two distinctly advantageous techniques for live cell imaging of the fluorescent biosensor include fluorescence microscopy and flow cytometry. For the former, microscopy allows for direct visualization of cell morphology, for tracking cellular fluorescence over time, and for monitoring spatial dynamics of fluorescence (Fig. 2a). Generally, any fluorescence microscope with power to resolve individual bacterial cells can be used, but analysis of results with the

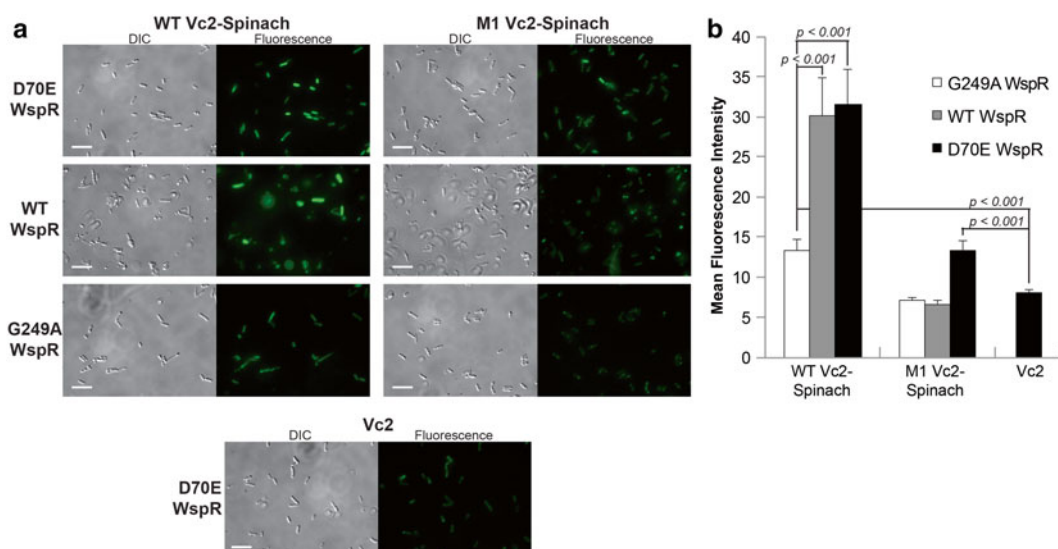


Fig. 2 The Vc2-Spinach biosensor detects cdiG through fluorescence microscopy of live *E. coli*. **(a)** Differential interference contrast (DIC) and fluorescence microscopy images are shown of representative *E. coli* expressing variants of the Vc2-Spinach biosensor and the WspR cdiG synthase. Scale bars represent 10 μ m. **(b)** Quantitation of mean fluorescence intensity from fluorescence microscopy experiments. Error bars represent the SEM for at least 50 quantified cells. Indicated *p* values were calculated using a student's *t*-test. Figure used with permission since data is from JACS paper (reference)

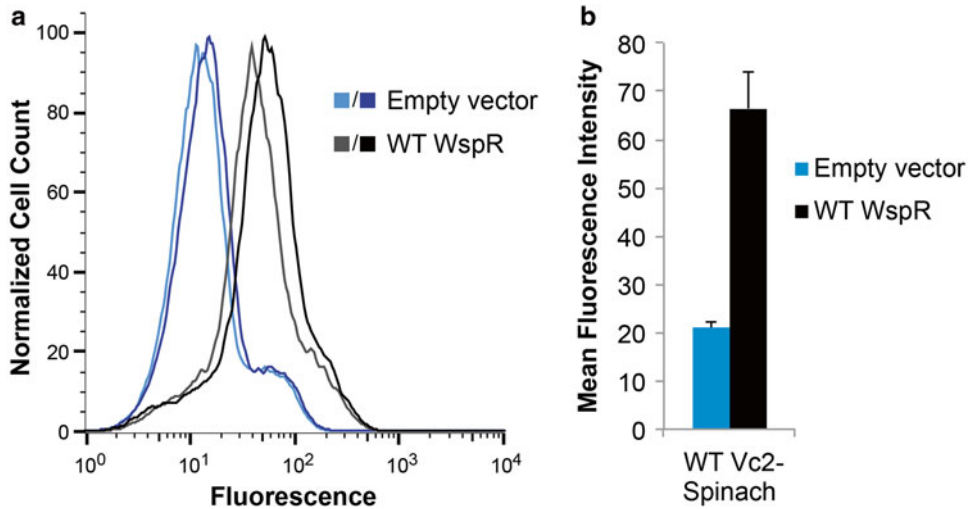


Fig. 3 The Vc2-Spinach biosensor detects *cdiG* through flow cytometry of live *E. coli*. **(a)** Flow cytometry data of cells coexpressing the WT Vc2-Spinach biosensor along with empty vector or WT WspR. **(b)** Quantitation of mean fluorescence intensity from flow cytometry experiments. Error bars represent the standard deviation of independent biological replicates of at least 35,000 cells

currently available software is time-consuming. On the contrary, flow cytometry or fluorescence activated cell sorting (FACS) allow for rapid and high-throughput analysis of thousands of cells (Fig. 3a). These flow-based methods provide a snapshot of bacterial characteristics of a large population at a specific time, and the ability to sort cells exhibiting differential fluorescent outputs. However, flow techniques are not suited for tracking the behavior of individual cells over time. Nevertheless, both experimental techniques have proven robust and reproducible using RNA-based biosensors.

3.2.1 Preparation of Poly-D-Lysine Coverslips for Fluorescence Microscopy

In performing live cell fluorescence microscopy experiments, the coverslips used for adhering the cells are first prepared and can be stored at room temperature for several months. Cells will adhere to the poly-lysine coated coverslips through electrostatic interactions between the positive charge of the lysine residues on the coverslips and the negative charge of the bacterial cell. Specially designed plates and other poly-lysine coated materials can alternatively be ordered from commercial vendors such as MatTek.

Step 7: Acid rinse of coverslips (Estimated time: 11 h)

1. To coat the coverslips with a negative charge, soak an adequate number of coverslips in 1 M HCl and heat the solution to $\sim 50^\circ\text{C}$ for 1 h. Let the solution cool to ambient temperature for 2–10 h with occasional mixing.

2. Carefully remove the coverslips from the acid solution, then rinse the coverslips twice with ddH₂O and once with 100 % ethanol. Let the coverslips dry at ambient temperature for at least 1 h. Take care to neutralize the acid solution and to follow proper waste disposal guidelines.

Step 8: Poly-D-Lysine rinse of coverslips (Estimated time: 11 h)

1. To coat the coverslips with poly-D-Lysine, dilute 100 μ L 4 $\times$ poly-D-Lysine solution with 300 μ L ddH₂O. Let the coverslips soak in this solution in a petri dish for 1–10 h on a rocker at ambient temperature.
2. Rinse the coverslips carefully twice with ddH₂O water and once with 100 % ethanol. Remove the coverslips from the petri dish and dry them on filter paper for at least 1 h at ambient temperature. Coverslips can be stored at room temperature for ~3 months.

**3.2.2 Fluorescent
Microscopy Experiments**

Step 9: Bacterial growth and induction conditions (Estimated time: 16–20 h)

1. Inoculate a 3 mL LB/Carb/Kan culture with a single colony of the transformed BL21 star cells. Incubate the culture at 37 °C in an incubator/shaker for 12–16 h or overnight.
2. In the morning, inoculate a fresh 3 mL LB/Carb/Kan culture with 200 μ L of overnight culture. Grow the cells in a 37 °C incubator shaker and monitor growth by measuring the OD₆₀₀.
3. Once the cells have reached an OD₆₀₀ 0.4–0.6 (approx. 2 h), induce the expression of the biosensor and plasmid with isopropyl β -D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. Continue growing the cells in a 37 °C incubator shaker for 2.5 h.

Step 10: Harvesting cells and preparation of slides (Estimated Time: 3.5 h)

1. To collect cells and remove LB, pellet 100 μ L cells at 4,500 rcf for 3 min at ambient temperature. Remove the supernatant and resuspend the cell pellet in 500 μ L M9 minimal media, pH 7.0. Wash the cells of LB by centrifuging again, removing the supernatant, and resuspending in another 200 μ L M9 minimal media, pH 7.0 (*see Note 6*).
2. Adhere the suspended cells to the prepared poly D-Lysine coverslips by pipetting the cells onto each coverslip and allow the cells to attach for ~30 min at 37 °C.
3. To remove any unadhered cells, gently rinse the coverslips with 3 mL M9 minimal and aspirate off the solution.
4. Add 200 μ L of 200 μ M DFHBI in M9 minimal media, pH 7.0 to the cells and incubate the cells at 37 °C for ~1–1.5 h to

allow DFHBI to permeate the membrane and to bind the biosensor RNA. Keep the cells in the dark from this point on as DFHBI is light-sensitive.

5. Remove excess liquid by aspiration and place the coverslip on top of a microscope slide. Seal the edges of the coverslip with nail polish to prevent drying of the cells.

Step 11: Imaging cells using fluorescence microscopy (Estimated time: 1 h)

1. To image cells via microscopy, first place the brightest expected sample on the microscope platform and focus on the cells using bright-field imaging. Determine the fluorescence exposure to be used by maximizing the exposure time to increase fluorescence signal without overexposing any cells (*see Note 7*). Note this fluorescence exposure setting and revert to bright-field exposure while fluorescence imaging is not active.
2. Image all samples by first taking a snapshot of cells in the bright field, then switch to green fluorescence filters using the previously determined exposure time and take a snapshot of the same field visualized by fluorescence. For each sample, repeat this procedure for at least three different viewing sections, where at least a total of 50 individual cells can be visualized. Follow this procedure for all samples of interest and save all images as files that can be opened in ImageJ (e.g., tif files).

Step 12: Analysis of fluorescence microscopy data (Estimated time: 3 h)

1. To normalize all microscope images to the brightest sample of the set, open the fluorescent tif file in ImageJ of the brightest sample tested. In the adjustment category of the image menu, select Brightness/Contrast and set it to Auto. Note the maximum displayed value of the 8-bit image as this will be used to set the maximum brightness of all images.
2. To determine the background fluorescence of each sample, which varies based upon the amount of DFHBI left on the coverslip, outline at least four areas of the image that contain no cells and use the measurement tool to determine the median fluorescence for each area. The average median fluorescence of the four areas (typically ± 2 units within the minimum fluorescence set by the Auto function) is the background fluorescence of the image.
3. Adjust each image individually by setting the minimum displayed value to the background fluorescence found in **step 2**, and the maximum displayed value to the maximum brightness found in **step 1**. Click ok and then apply within the Brightness and Contrast tool bar. Complete this process for all images to be analyzed.

4. To determine the mean fluorescence of each cell, individual cells will be manually outlined in the bright-field image and then overlaid on the corresponding fluorescence image. Open the bright-field image in ImageJ then manually outline each cell using the heart-shaped freehand selection tool. Hold down the Shift key while outlining to keep all previously outlined cells selected and periodically save the outlined cells as regions of interest (ROIs). If at any point an error is made during this process, click on the background of the image to clear the mistake, then select the last object in the ROI manager to redo that step.
5. Once all cells in the bright-field image have been outlined, delete all ROI entries except for the last object containing all outlined cells. Open the fluorescence image, then select the object in the ROI manager to have the outlines appear on this image. Some adjustment may be necessary if the camera shifted between taking snapshots of the bright-field and fluorescence images. If so, simply move the ROI selections using the arrow keys. Save the image as a separate file by overlaying the ROI selections from the ROI manager.
6. Obtain the median fluorescence for each outlined cell by selecting to Split the combined object in the ROI manager. This separates each outlined object, and the fluorescence statistics can be obtained by highlighting the individual cells in the ROI manager and selecting Measure.
7. Record the obtained cell statistics and also measure the background fluorescence of four areas of the image that have no cells. Determine the mean fluorescence intensity of each cell by subtracting this background from the mean fluorescence of each cell. For each sample tested, determine the fluorescence of at least 50 cells to calculate the average mean fluorescence intensity for this sample. Assess the statistical significance of the differences in sample fluorescence using a student's *t* test (Fig. 2b).

3.2.3 Flow Cytometry Experiments

Step 13: Growth of cells for flow cytometry (Estimated: 16 h)

1. To prepare cells for flow cytometry, follow **step 9** as discussed in Subheading 3.2.2 (*see Note 8*).

Step 14: Preparation of cells for flow cytometry (Estimated time: 1 h)

1. To collect cells and remove LB, pellet 250 μ L cells at 4,500 rcf for 3 min at ambient temperature. Remove the supernatant and resuspend the pellet in 500 μ L 1 $\times$ PBS (*see Notes 6 and 9*).
2. Add 1–5 μ L of the suspended cells to 250 μ L of the 25 μ M DFHBI in 1 $\times$ PBS. Assuming that OD₆₀₀ = 1 corresponds with approximately 1×10^9 cells/mL, this should give a final concentration of between 3,000 and 15,000 cells/ μ L.
3. Filter the suspension through the cell-strainer caps into polypropylene tubes for analysis.

Step 15: Analysis of cells by flow cytometry (Estimated time: 1 h)

1. For future analysis of cells via flow cytometry, establish the appropriate gating settings using both positive and negative fluorescence controls. To do this, first establish the forward scatter (FSC) and side scatter (SSC) regions corresponding to cells by first analyzing DFHBI-PBS solution alone, followed by a solution containing cells in DFHBI-PBS solution.
2. To optimize the voltage gain settings, pass the negative control—either cells expressing no dinucleotide cyclase or a non-functional biosensor—through the analyzer, and compare to the positive control with cells coexpressing the Vc2-Spinach biosensor with WT WspR.
3. Analyze all samples by collecting data for at least 10,000 cells within the gating window.

Step 16: Analysis of flow cytometry data (Estimated time: 1 h)

1. Using FlowJo software, generate a gate from the FSC/SSC dot plot to analyze data points solely corresponding to cells and limiting the amount of debris analyzed. Apply this same gate to all samples tested.
2. From this gate, use the FlowJo statistical analysis to determine the mean fluorescence intensity of each sample. Calculate the standard deviation from the mean fluorescence intensity of at least two independent biological replicates (*see* Fig. 3b).

4 Notes

1. To use the biosensor to analyze the effect of an enzyme on metabolite levels, it is advisable to compare cells harboring both biosensor and enzyme versus cells harboring both biosensor and empty vector. The latter control eliminates variation in expression caused by cells grown under different antibiotic conditions and with different plasmid loads.
2. As described in Subheading 3.1, other pET vectors may be used, but it is recommended to use compatible plasmids if co-expressing the biosensor with an enzyme.
3. This protocol describes the use of the Spinach system with the original Spinach aptamer and fluorophore DFHBI. An improved version of Spinach, termed Spinach2, and alternative fluorophores, DFHBI-1T and DFHBI-2T, have recently been developed for in vivo analysis [15, 16]. The Spinach2 aptamer has increased folding stability and displays 2.1-fold and 3.2-fold increases in fluorescence in bacterial and mammalian cells, respectively.
4. The Vc2-Spinach DNA template can either be ordered as a DNA oligo from a commercial vendor or can be generated through overlapping PCR [9].

5. Amplification of genes with high GC-content such as WspR can be difficult. If necessary, 1.5–3 μL of DMSO can be added to the PCR amplification. Alternatively, consider using a different enzyme gene that would suit the experiment.
6. One of the most important parameters with both microscopy and flow cytometry analyses is to ensure that the DFHBI solution is the same concentration across all samples. We generally make the 1 $\times$ PBS or M9 Minimal Media solution containing DFHBI fresh for each set of experiments.
7. If no fluorescence is observed, yet the biosensor has been confirmed to function *in vitro*, examine the effect of the tRNA scaffold on fluorescence *in vitro*. Alternatively, test whether the Spinach-tRNA construct alone functions *in vivo* to assess if the problem is due to RNA expression, DFHBI permeability, or the microscopy protocol. If Spinach expression can be visualized, consider that ligand concentration might be too low for fluorescence activation or other effects of ligand on fluorescence visualization. If Spinach expression cannot be visualized, check that the RNA is being expressed via Northern blot or RT-PCR analysis.
8. Flow cytometry experiments can also be carried out by growing and analyzing cells in 96-well plate format. In a 1,000 μL deep-well plate, inoculate 200 μL of LB/Carb/Kan media with 5 μL of overnight culture and incubate the cells in a 37 °C shaker for ~1.5 h. Induce the cells with 1 mM IPTG, then harvest the cells after ~3 h incubation by centrifuging the entire plate at 4,500 rcf for 3 min at ambient temperature. Decant the supernatant and resuspend the cells in 500 μL 1 $\times$ PBS. Add 1 μL resuspended cells to 150 μL of the DFHBI-PBS solution in a 96 well plate and read using a BD High Throughput Sampler.
9. We have found that both M9 minimal media, pH 7.0 and 1 $\times$ PBS are suitable for preparing cells for flow cytometry. Due to the longer incubation for adhering cells in the microscopy procedure though, we recommend using M9 minimal media for microscope experiments.

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Chapter 9

RNA Scaffold: Designed to Co-localize Enzymes

ZJU_China Team (iGEM 2012) and Ming Chen

Abstract

Self-assembling RNA scaffold is designed to co-localize enzymes in engineered biological pathways through interactions between scaffold's protein docking domains and their affinity protein–enzyme fusions, *in vivo*. Here we introduced a noncoding RNA structure theophylline aptamer that respond to theophylline ligand in order to modulate RNA scaffold and regulate multistep enzymatic activities. We described the specifically designed RNA scaffold increased the fluorescent intensity in a splitting GFP assay by 2.25-fold, and also observed a 1.43-fold increase in multi-enzymatic efficiency in IAA synthesis pathway.

Key words RNA scaffold, Aptamer, Riboswitch, Splitting GFP, Co-expression, Overlap PCR

1 Introduction

Spatial organization in bacterial metabolism is crucial for reaction efficacy by increasing reaction yield and limiting pathways cross talk [1, 2]. Self-assembling RNA scaffolds can be designed to engineer enzymes spatial organization, and allows efficient channeling of substrates to products over several enzymatic steps by limiting the diffusion of intermediates. Compare to traditional DNA scaffolds and protein scaffolds, rationally designed RNA scaffolds are more flexible to regulate the enzyme co-localization. Basic RNA scaffold designed to co-localize enzymes was first established by Delebecque et al. [3]. The basic RNA scaffold consists of two protein-docking RNA aptamers (MS2 aptamer and PP7 aptamer, these are considered as expression aptamer platforms that have affinity to MS2 aptamer protein and PP7 aptamer protein, respectively). Here we designed a particular RNA scaffold that combines a modified noncoding allosteric RNA aptamer that can be modulated by theophylline induction, and the basic RNA scaffold structure [4]. Previous studies have revealed the switch nature of theophylline aptamer (a single RNA hairpin structure that has high affinity to theophylline in its inner loop region) and a structural

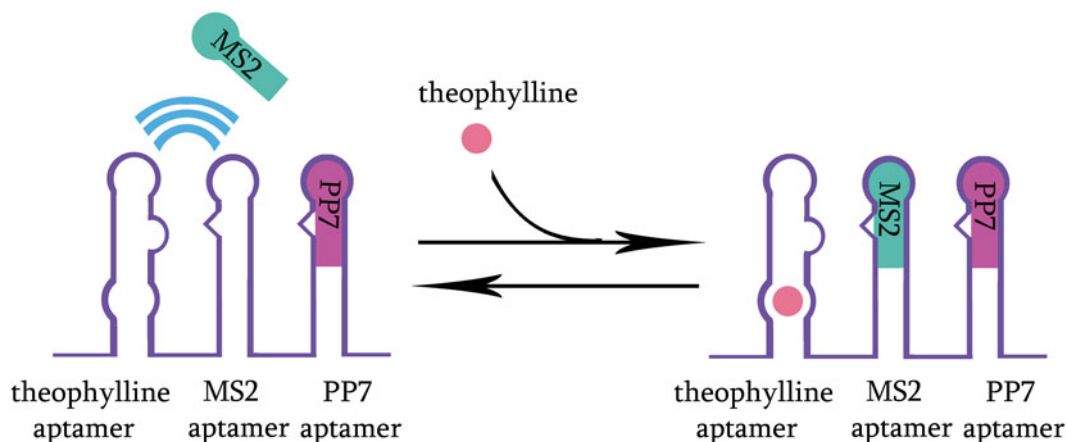


Fig. 1 When unbounded, theophylline aptamer is engineered in a way that the loop region will interact with MS2 aptamer protein binding site, therefore inactivates MS2 aptamer. When bounded, theophylline aptamer will be structurally modulated. Such ligand binding and structure modulation eliminate the structural interactions therefore activates MS2 aptamer

interaction between unbound theophylline aptamer and expression aptamer platform (in this case the unbound theophylline aptamer was modified to interact with MS2 aptamer protein binding site) (*see* Fig. 1)

In order to characterize the system, this method adapts a GFP splitting technique to test the regulation of RNA scaffolds co-localization [5, 6]. Two halves of GFP genes (Fragment A as FA, and Fragment B as FB) each fused to MS2 or PP7 by overlap extension PCR cloning, then inserted to three plasmids compatible to each other (pACYCDuet-1, pCOLADuet-1 and pETDuet have different cloning mechanisms). Co-expression of pACYCDuet-1-FA-MS2, pCOLADuet-1-FB-PP7, and pETDuet-scaffold vectors will better enable two protein fusions bind to self-assembled RNA scaffold with the presence of theophylline ligand, then FA and FB reconstruct to a functional GFP and fluorescent.

This technique combines regular PCR cloning, overlap PCR, TA cloning, plasmids co-expression and other basic genetic engineering methods. Similar principles can be utilized for designing versatile RNA scaffolds that serve multiple purposes in constructing nanoscale architecture. For the purpose of enzyme co-localization, FA and FB can be replaced by other enzymes. MS2 protein and PP7 protein can be genetically modified that each links to an individual enzyme in the designated pathway. We have demonstrated a 1.43-fold acceleration in IAA biosynthesis pathway by organizing enzymes' subcellular localization.

2 Materials

2.1 Equipment

1. NanoDrop ND-1000.
2. UV transilluminator.
3. Olympus FLUOVIEW FV1000 confocal laser scanning microscope.
4. Automated thermal cycler.

2.2 Supplies and Reagents

1. Milli-Q water.
2. DNA ladder Quick-Load® 2-Log DNA Ladder (0.1–10.0 kb) purchased from New England BioLabs® Inc.
3. Pfu Mix.
4. 10,000× Gel Red Nucleic Acid Gel Stain.
5. rTaq polymerase.
6. Restriction enzymes: HindIII, NcoI, NdeI, XhoI.
7. 10× Buffer Tango.
8. 1 M IPTG: Weigh 2.4 g, add 10 mL water, sterilize with 0.22 µm filter, and freeze in aliquots.
9. 20 mg/mL X-gal stock solution: Weigh 0.2 g X-gal, dissolve in 10 mL *N,N*-Dimethylformamide (DMF). Store at –20 °C (*see Note 1*).
10. Stock antibiotic solutions: 100 mg/mL ampicillin (weigh 1 g ampicillin, add 10 mL water, sterilize with 0.22 µm filter and freeze in aliquots), 50 mg/mL kanamycin (weigh 0.5 g kanamycin, add 10 mL water, sterilize with 0.22 µm filter and freeze in aliquots), 10 mg/mL spectinomycin (weigh 0.1 g spectinomycin, add 10 mL water, sterilize with 0.22 µm filter and store in –20 °C in aliquots).
11. LB broth: Dissolve 5 g tryptone, 2.5 g yeast extract, and 5 g NaCl in 450 mL nuclease-free water in a 500 mL Erlenmeyer flask or a 500 mL glass bottle. Measure the pH by using pH meter, then adjust to pH 7.0 (*see Note 2*), then add water to a volume of 500 mL. Autoclave on liquid cycle for 20 min at 15 psi. Store at room temperature.
12. LB agar plates: Prepare LB broth as in previous step, and dissolve 7.5 g Agar. Autoclave on liquid cycle for 20 min at 15 psi. After autoclaving, cool it to about 60 °C (in room temperature). If necessary, add appropriate amount of antibiotic stock solution through filter sterilization unit (50 µg/mL ampicillin, 25 µg/mL spectinomycin, 25 µg/mL kanamycin).
13. 50× TAE buffer: Weigh 242 g Tris base and 37.2 g Na₂EDTA·2H₂O, add 57.1 mL glacial acetic acid then add water to 1 L.

14. 0.1 % agarose gel: Dissolve 0.2 g agarose powder in 20 mL 1× TAE buffer in a 100 mL flask. Microwave the solution for 1 or 2 min until fully dissolved. Let the flask cool to about 60 °C, and then add 2 µL of 10,000× Gel Red, swirling the flask until fully mixed. Seal both ends of the casting tray with tapes and place a 10-well gel comb on one side. Carefully pour the solution into the tray without introducing any air bubbles. Let it cool for 30 min until solid. Carefully remove the tapes and pull out the combs.
15. 6× sample loading buffer: 10 mM Tris–HCl (pH 7.6), 0.03 % bromophenol blue 60 % glycerol 60 mM EDTA.
16. 50 % glycerol stock: Mix 20 mL glycerol and 20 mL water (*see Note 3*), autoclave on liquid cycle for 20 min at 15 psi. Store at 4 °C.
17. 1 M theophylline solution: Weigh 1.8 g theophylline, add 10 mL water, sterilize with 0.2 µm filter, and store in –20 °C in aliquots.
18. Phosphate buffered saline (PBS): Weigh 8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄, 1.8 g K₂HPO₄, mix and dissolve in 800 mL water, adjust pH to 7.4 with HCl. Add water to 1 L. Autoclave on liquid cycle for 20 min at 15 psi. Store at room temperature.

3 Methods

Carry out all procedures on ice unless otherwise specified.

3.1 Recombinant Plasmids Construction (e.g., pACYCDuet-1-FA-MS2)

3.1.1 Gene Amplification by PCR and Gel Electrophoresis

1. Design appropriate primers (*see Note 4*). A, B, C, D including restriction enzyme site and overlap fragment based on the sequences of half EGFP (FA, fragment A) and MS2 aptamer (*see Fig. 2*).
2. Amplify FA gene and MS2 gene individually. Prepare a total volume 10 µL PCR reaction solution in sterile PCR tubes by mixing 5 µL Pfu Mix, 0.1–10 ng target DNA template, 0.25 µL Primer Forward and 0.25 µL Primer Reverse, then add nuclease-free water until 10 µL.
3. Vortex the PCR tubes for 5 s, then spin all the liquid down for 5 s.
4. Start PCR amplification with the following cycles: (1) 94 °C 5 min, (2) 30 cycles of 94 °C 20 s, 55 °C 20 s and 72 °C 1 min. (3) 72 °C 5 min. (4) 4 °C rest.
5. Spin all the liquid down for 5 s, and then place the PCR tubes back on ice.
6. Prepare a 0.1 % agarose gel. Place the gel in the electrophoresis chamber (arrange the gel so the side has wells is on the cathode

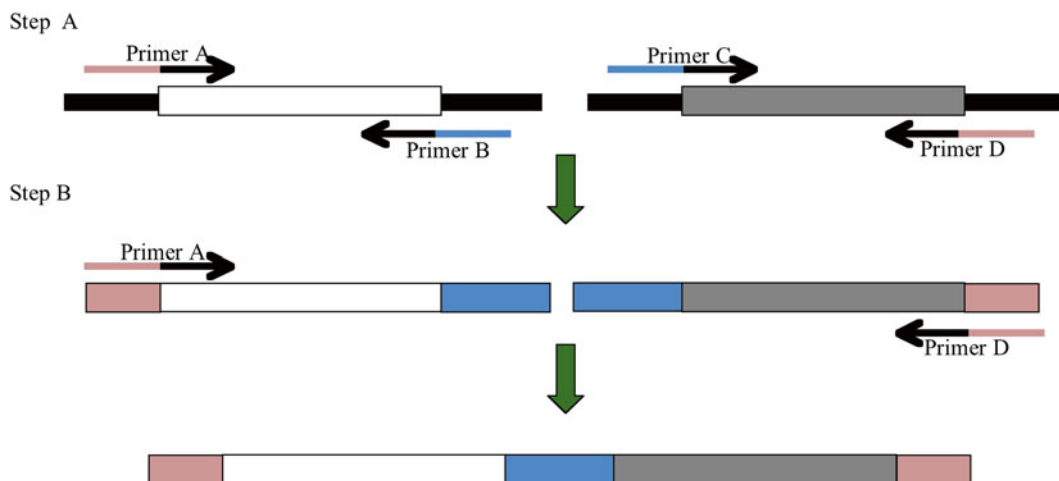


Fig. 2 Overview of overlap PCR. (Step A) Design two pairs of primers A + B, C + D. Amplify enzyme gene and aptamer gene respectively with PCR. (Step B) Take amplified DNA fragments as template, add primers A and D to perform PCR. Due to the overlap region, the two fragments will be combined together after PCR (*red* parts refer to restriction enzyme sites, *blue* parts refer to overlap fragments, *white* parts refer to enzyme genes and *grey* parts refer to aptamer genes)

side of chamber), add 1× TAE buffer until there's about 2 mm of buffer over the gel surface.

7. Add 2 μL 6× sample loading buffer into sample PCR tubes (10 μL) and mix by pipetting up and down. Carefully load all solutions into each well in the gel. Record the well number and corresponding sample name.
8. Pipette 10 μL of the DNA ladder standard into the first well and/or last well (if applicable).
9. After loading the samples, place the lid back on the electrophoresis tank; connect it to the power supply. Set the voltage to 100 V, and set the time to 20 min (or until the tracing dye reach two thirds of the gel length).
10. Visualize the gel under UV light. Cut the stripe that contains the target size band, and then perform DNA gel extraction with Kit.
11. Measure the DNA concentration via NanoDrop.
12. Store the DNA at $-20\text{ }^{\circ}\text{C}$ if not use it immediately.

3.1.2 Enzyme-Aptamer Linking by Overlap PCR

1. Prepare a total volume 10 μL overlap PCR reaction solution in sterile PCR tubes by mixing 5 μL Pfu Mix, 0.5 μL target enzyme DNA template, 0.5 μL target aptamer DNA template, 0.25 μL primer A and 0.25 μL primer D (*see Fig. 1*), then add nuclease-free water until 10 μL .
2. Repeat **step 3–12** in Subheading 3.1.1.

3.1.3 Construct Plasmid by TA Cloning

1. Add a single 3'-adenine overhang by pretreating DNA in sterile PCR tubes: mix 5 μ L DNA PCR gel purified product and 5 μ L rTaq polymerase.
2. Pipette the solution up and down without introducing air bubbles, and then incubate the tubes at 72 °C for 15 min.
3. Ligate the pretreated DNA fragment with T-vector by preparing a total volume 10 μ L ligation reaction solution in sterile PCR tubes: mix 4 μ L pretreated DNA sample, 5 μ L Solution 1, 1 μ L T-Vector, then incubate in 15 °C for 3 h (or overnight).

3.1.4 Transformation and Antibiotic Screening

1. For each transformation reaction, thaw 80 μ L Top 10 competent cells on ice for about 20 min. Add 5 μ L Sequencing plasmid. Mix it by gently pipetting up and down for a few times. Then place on ice for 30 min.
2. Warm up antibiotic LB agar plates to room temperature.
3. Heat-shock each transformation reaction tube by placing into 42 °C water bath for 45 s. Then immediately place the tubes back on ice for 2 min.
4. Add 500 μ L LB broth to the reaction tube, gently mix it by pipetting. Then place in 37 °C shaking incubator for 1 h.
5. Centrifuge the reaction tube at maximum speed for 30 s. Dump the majority of supernatant and gently resuspend the remaining solution.
6. Putting IPTG and X-gal on top of premade agar plates. Spread 40 μ L IPTG and 40 μ L X-gal on top of the plate with a hockey stick spreader. Then, let the plates dry before using them. This should take 30 min or so.
7. Plate 10 μ L of resuspended cells on prepared LB agar plate.
8. Incubate plates at 37 °C for 8 h (or overnight).

3.1.5 Plasmid Extraction and Sequencing

1. Pick the white colonies from plates and grow in 4 mL antibiotic LB broth overnight.
2. Extract the plasmid with miniprep kit.
3. Measure the DNA concentration via NanoDrop.
4. Send the plasmid sample to get sequenced.

3.1.6 Double Digestion and Ligation

1. Prepare a total volume 20 μ L double digestion reaction solution in sterile PCR tubes by mixing 1 μ L HindIII, 0.5 μ L NcoI, 2 μ L 10 $\times$ Buffer Tango, 8 μ L correct plasmid, and pACYCDuet-1. Incubate at 37 °C for 2 h (*see Note 5*).
2. Repeat **steps 6–14** in Subheading **3.1.1**.
3. Ligate the extracted DNA fragments by preparing a total volume 20 μ L ligation reaction solution in sterile PCR tubes: mix 17 μ L extracted DNA sample (vector : insert $\approx$ 1:5), 2 μ L 10 $\times$ Ligation

Buffer, 1 μ L T4 DNA ligase, add nuclease-free water until 20 μ L. Vortex the PCR tubes for 5 s, then spin all the liquid down for 5 s. Incubate at 16 °C for 3 h (or overnight).

4. For Transformation and antibiotic screening, repeat Subheading 3.1.4.
5. For Plasmid extraction and sequencing, repeat Subheading 3.1.5.
6. Construct another two plasmids pCOLADuet-1-FB-PP7 and pETDuet-scaffold using similar methods listed above.

3.1.7 Co-transformation and Co-expression (Split GFP Experiments)

1. Mix pACYCDuet-1-FA-MS2, pCOLADuet-1-FB-PP7, and pETDuet-scaffold, transform the mixed plasmids to BL21*(DE3) (*see Note 6*) according to Subheading 3.1.4.
2. Pick single colony from the plate. Grow in 5 mL antibiotic LB (50 μ g/mL ampicillin, 25 μ g/mL spectinomycin, 25 μ g/mL kanamycin).
3. Induce the cells by 0.2 M IPTG and add a certain concentration of theophylline for 2 h at mid-log phase (*see Note 7*).
4. Wash the cells twice with equivalent PBS.
5. Then test the Fluorescence intensity (FI) and OD with Biotek Synergy Hybrid Reader (*see Fig. 3*). Take picture with Confocal Scanning Microscope.

4 Notes

1. X-gal solution must be wrapped in aluminum foil to protect it from the light, and stored in the freezer. X-gal solutions do not need to be sterilized by filter, because the filter membrane will be dissolved by DMF.
2. pH influences the growth rate of *E. coli*. We adjust LB broth to pH 7.0 by first measuring the original pH, then add 0.1 M NaOH or 0.1 M HCl drop wise until the reading stabilize at pH 7.0.
3. Use large stir bar and magnetic stir plate to fully mix the 50 % Glycerol stock solution until the mixture is homogeneous.
4. When designing primers, add protective nucleotides flanking restriction sites improves digestion efficiency. Primer design for overlap PCR involves the addition of an overlap region. It is a repeat sequence (GGAGGAGGAGGATCAGGAGGAGGAGGATCA) that serves as a linker to connect two protein domains without interfering with their respective functions (e.g., FA is fused to MS2 with the linker in between).
5. Proper buffer can be chose to optimize the reaction conditions for double digestion efficiency. We used the Double Digest tool (<http://www.thermoscientificbio.com/webtools/doubledigest/>) provided by Thermo Scientific to calculate the recommended buffer for selected restriction enzymes.

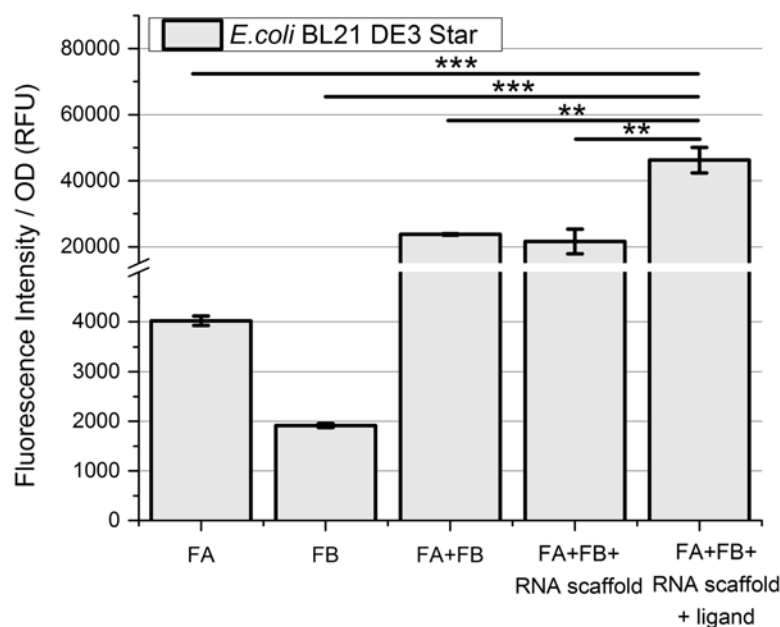


Fig. 3 Fluorescence intensity of co-expression *E. coli* BL21 DE3 Star in splitting GFP assay. The data was obtained by Biotek Synergy H1 Hybrid Reader. Strain *E. coli* BL21*(DE3) were transformed with pACYCDuet-1-FA-MS2, pCOLADuet-1-FB-PP7, pETDuet-scaffold or designated plasmids combination. 0.2 M IPTG was used to induce expression in all conditions. 0.5 mM theophylline ligand solution was added in the three plasmids co-expression group. Ligand treated co-expression group demonstrate a significant increase (one way ANOVA, Tukey's Multiple Comparison Test, p -value < 0.001 denoted by *** p -value < 0.01 denoted by **)

- The most important feature of BL21*(DE3) is that it carries a mutated *rne* gene (*rne131*) which encodes a truncated RNase E enzyme that lacks the ability to degrade mRNA, resulting in an increase in mRNA stability.
- When induced by 0.2 M IPTG, relatively low temperature (20 °C) will help to properly fold the protein three dimensional structures thus prevent the formation of aggregation.

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Artificial Ligase Ribozymes Isolated by a “Design and Selection” Strategy

Shigeyoshi Matsumura and Yoshiya Ikawa

Abstract

We developed a new in vitro selection strategy “design and selection” to isolate effectively artificial ribozymes (catalytic RNAs). An overall RNA structure (scaffold) is initially designed, and then a relatively short randomized sequence is installed at the reaction point of the scaffold, followed by the in vitro selection. This method can reduce the length of randomized sequence, providing large coverage of the sequence space in contrast with the conventional way, which makes the selection experiment effectively. Additionally, further analysis of ribozymes obtained by this approach is practically easy since the overall molecular structure is predesigned and well known. Here we show the procedure to isolate artificial RNA ligase ribozymes by this strategy. We have succeeded in isolation of the *designed and selected ligase* (DSL) ribozymes.

Key words Ribozyme, RNA ligase, Rational design, Structural motif, In vitro selection

1 Introduction

RNA is an enormously versatile biomolecule capable of performing variety of tasks, e.g., binding and catalysis. Many catalytic RNAs (ribozymes) have been found in nature, including a group I and II self-splicing intron, bacterial RNase P, ribosome, and hammerhead, but most of such natural ribozymes catalyze exchange of RNA phosphodiester bond. Catalytic potential of RNA has been largely expanded by in vitro selection technique generating plenty of unnatural ribozymes. This technique was invented by mimicking natural Darwinian evolution in vitro [1]. For instance, artificial RNA ligase ribozymes joining two RNA strands were obtained by many researchers [2–5].

The in vitro selection technique, however, has a critical drawback, that is coverage of sequence space. To form a catalytically active structure, certain length of RNA strand is demanded, typically >50 nucleotides (nt). Theoretically possible sequence variation of the 50 nt length RNA is 4^{50} ($\approx 10^{30}$), whereas amount of RNA that can be practically handled is limited (10^{15} to 10^{16} molecules).

It means that we are not able to survey vast majority of the sequence space. Most extreme case is the isolation of a highly active RNA ligase ribozyme by Bartel and Szostak [2]. They obtained the ribozyme from 220 nt-randomized sequence library whose sequence space is 10^{132} , examining only 10^{-114} % of the space. Experimental reproduction of the result is likely to be impossible since such selection experiment will depend on coincidence, making the scientific discussion complicated.

RNA structural biology and nanotechnology are currently advancing, leading rational construction of RNA structure. It is possible to design a new RNA scaffold by combining many RNA structural motifs which are available from huge structural data [6]. We developed a new strategy “design and selection” to isolate effectively artificial ribozymes [7]. An overall RNA structure (scaffold) is initially designed, and then a relatively short randomized sequence is installed at the reaction point of the scaffold, followed by the in vitro selection. First, this method can reduce the length of randomized sequence, providing large coverage of the sequence space which makes the selection experiment effectively. Second, since the overall molecular structure is predesigned and known, further analysis of the obtained ribozymes will be practically easy. Here we show the procedure to isolate artificial RNA ligase ribozymes by the “design and selection” strategy. We have succeeded in isolation of the designed and selected ligase (DSL) ribozymes.

2 Materials

2.1 Design of Self-Folding (Scaffold) RNA

1. Molecular modeling software, Discovery Studio (Accelrys).

2.2 Pool Synthesis

1. Synthetic DNA oligonucleotides.

For initial library

pool L-a: 5'-*GAA GTA ATA CGA CTC ACT ATT* AGG GAA GGA AAC TTC CCT GTG GAA ATT GCA ACT ACG GTT GCA GCG TAG TC-3', where T7 promoter sequence is shown in italics.

pool N30L-b: 5'-GAA ATT GCA ACT ACG GTT GCA GCG TAG TCT -N30- CCT AAG GCAAAC GCT ATG GAC TGA GAC-3', where N₃₀ indicates 30 random nucleotides.

pool L-c: 5'-TCT GCC TAA GTG GGC AAT GAG ACT GGA ACG CAG TCT CAG TCC ATA GCG TTT GCC TTA GG-3'.

pool R-a: 5'-*GAA GTA ATA CGA CTC ACT ATT* AGG GAA GGA AAC TTC CCT GTG GAA ATT GCA ACT ACG GTT GCA GCG TAG TCT CAG TCC TAA GGC AAA CGC TAT GG-3', where the T7 promoter sequence is shown in italics.

pool N30R-b: 5'-GTC TCA GTC CTA AGG CAA ACG CTA
TGG-N₃₀-AGA CTG CGT TCC AGT CTC ATT GCC CAC-
3', where N₃₀ indicates 30 random nucleotides.

pool R-c: 5'-TCT GCC TAA GTG GGC AAT GAG ACT GGA
ACG CAG TC-3'.

For doped library

KTL-1 dope sense: 5'-GAA GTA ATA CGA CTC ACT ATT AGG
GAA GGA AAC TTC CCT GTG TCA ATT GCA ACT ACG
GTT GCA GCG TAG TCN NNN NCC TAA GGC AAA
CGC TAT GG-3', N indicates a random nucleotide.

KTL-1 dope antisense: 5'-TCT GCC TAA GTG GGC AAT GAG
ACT GGA ACG CAG TCt gct ggg gaa cgc att aag cta ccc gtt
gat CCA TAG CGT TTG CCT TAG G-3', small letters cor-
respond to degenerate bases comprising 61 % of the original
base and 39 % of three other bases.

2. dNTPs solution (2.5 mM each). Store at -20 °C.
3. *Ex Taq* DNA polymerase (Takara Bio, Japan).
4. PCR thermal cycler Dice (Takara Bio, Japan).
5. Phenol saturated with TE (10 mM Tris pH 8.0, 1 mM EDTA).
Store at 4 °C protected from light.
6. Diethylether.
7. 3 M sodium acetate pH 7.0.
8. 100 % (v/v) ethanol.
9. 70 % (v/v) ethanol.

2.3 In Vitro Transcription (IVT)

1. 10× T7 transcription buffer: 400 mM Tris-HCl (pH 7.5),
150 mM MgCl₂, 50 mM DTT, 10 mM spermidine.
2. 10× NTP (12.5 mM each).
3. α-P³² GTP.
4. T7 RNA polymerase.
5. RQ I RNase-free DNase (Promega).
6. 10 M ammonium acetate.
7. Denaturing dye: 70 % formamide, 0.15 % XC, 0.2 % BPB.

2.4 Polyacrylamide Gel Electrophoresis

1. Glass plate.
2. Spacer (1 mm thickness).
3. Comb (6 wells, 1.5 cm each).
4. 5× TBE buffer.
5. 30 % acrylamide-bis solution (29:1).
6. Urea.
7. 20 % (w/v) ammonium persulfate (APS).

8. *N,N,N,N'*-Tetramethylethylenediamine (TEMED).
9. Gel electrophoresis apparatus.
10. Electrophoresis power supply.
11. Thin layer chromatography plates F254 (20×20 cm, Merck).
12. UV lamp (UVP, USA).
13. RNA elution solution: 0.3 M NaOAc, 0.1 % SDS.
14. UV spectrophotometer (Beckman, USA).

2.5 *In Vitro* Selection

1. Synthetic DNA oligonucleotides.
 Rv-S: 5'-TCT GCC TAA GTG GGC AAT GAG ACT GG-3'.
 Rv-L: 5'-TCT GCC TAA GTG GGC AAT GAG ACT GGA ACG C-3'.
 Rv-M: 5'-TCT GCC TAA GTG GGC AAT GAG ACT GGA AC-3'.
 Primers for selective cDNA amplification:
 5'-CGT ACA CGT ACT CAC GCG TAT ACA GTC-3' for the S-1 substrate, and 5'-ACT TCC GAG CTG TAG AGT TAG CAG CGA-3' for the S-2 substrate.
 Primer for the guide sequence of S-1 substrate: 5'-*GAA GTA ATA CGA CTC ACT ATT* AGG GAA GGA AAC TTC CCT GTG GAA ATT G-3', T7 promoter and the internal guide sequence are indicated with italics and underline, respectively.
 Primer for the guide sequence of S-2 substrate: 5'-*GAA GTA ATA CGA CTC ACT ATT* AGG GAA GGA AAC TTC CCT GTG TCA ATT G-3', T7 promoter and the internal guide sequence are indicated with italics and underline, respectively.
2. Synthetic RNA oligonucleotides.
 S-1 substrate: 5' biotin-CGU ACA CGU ACU CAC GCG UAU ACA GUC CAC-3'.
 S-2 substrate: 5' biotin-ACU UCC GAG CUG UAG AGU UAG CAG CGA CAC-3'.
3. 1 M Tris-HCl (pH 7.7).
4. 1 M MgCl₂.
5. Streptavidin paramagnetic particles (Promega).
6. ReverTra Ace (Toyobo, Japan).
7. 150 mM KOH.
8. 150 mM HCl.
9. *Ex Taq* DNA polymerase (Takara Bio, Japan).

2.6 *Isolation of Individual Ribozymes*

1. pGEM-T vector (Promega).
2. Thermo Sequenase™ Primer Cycle Sequencing Kit (GE Healthcare, USA).
3. Automated DNA sequencer, 4300 DNA Analyzer (LI-COR, USA).

2.7 Ribozyme Activity Assay

1. Precise heat block or PCR thermal cycler.
2. 1 M Tris-HCl (pH 7.7).
3. 1 M MgCl₂.
4. 1 M KCl.
5. Stop solution: 85 % formamide, 100 mM EDTA, 0.1 % XC.
6. Bio-Imaging Analyzer BAS2500 (Fuji Film, Japan).

3 Methods

3.1 Design of a Self-Folding (Scaffold) RNA In Silico

1. Obtain a pdb file of the *Tetrahymena* group I intron P4-P6 RNA crystal structure (PDB ID: 1GID) from the RCSB Protein Data Bank database (<http://www.rcsb.org/pdb/home/home.do>).
2. Import the file into the Discovery Studio molecular modeling software.
3. Extract three motifs (GAAA tetraloop, 11 nt receptor, and triple-helical scaffold) from the P4-P6 RNA structure.
4. Align and assemble the three motifs on the software by connecting them with standard double helices to form a self-folding RNA.

3.2 Construction of Initial Pool

The 30 random nucleotides sequence is inserted into the L or R region in the P3a helix of the self-folding RNA. The two initial DNA pool containing 30 random nucleotides are constructed by PCR with three synthetic oligonucleotides.

1. The three oligonucleotides for constructing the pool L containing 30 random nucleotides in the L region were pool L-a, pool N30L-b, and pool L-c. The oligonucleotides for the pool R were pool R-a, pool N30R-b, and pool R-c.
2. In a total volume of 6 mL mix the following components: PCR templates (pool N30L-b or pool N30R-b) 20 nM, primers (pool L-a and pool L-c, or pool R-a and pool R-c) 500 nM, dNTP 0.2 mM, *Ex Taq* reaction buffer 1×, and *Ex Taq* DNA polymerase 150 U. Aliquot PCR reaction into sixty 0.5-mL tubes (100 µL each).
3. Perform 15 PCR cycles with a PCR thermal cycler by a following program: 94 °C 1 min, 60 °C 1 min, 72 °C 1 min. The PCR with 120 pmol of the pool N30L-b or pool N30R-b produces ≈200 pmol PCR products.
4. Collect the PCR products into a tube. To purify it, add 6 mL TE-saturated phenol, vortex strongly, and centrifuge for 5 min at 4,500×g. Transfer the aqueous phase to a new tube. Add 10 mL diethylether, vortex, and centrifuge as previously. Remove the diethylether phase.

Table 1
Selection conditions

Round	RNA (pmol)	Substrate/P1	Reaction time	MgCl ₂ (mM)	KCl (mM)
Initial selection					
1	2,000	S-1/type 1	18 h	50	200
2	340	S-2/type 2	18 h	50	200
3	500	S-1/type 1	2 h	50	200
4	200	S-2/type 2	1 h	25	50
5	200	S-1/type 1	5 min	25	50
Doped (second) selection					
1	800	S-2/type 2	18 h	50	200
2	300	S-1/type 1	1 h	50	200
3	300	S-2/type 2	5 min	25	50
4	300	S-1/type 1	1 min	25	50

5. Add 0.6 mL 3 M sodium acetate and 15 mL 100 % ethanol. Incubate for 20 min at -20°C and centrifuge for 20 min at $4,500\times g$ at 4°C . Remove supernatant, add 20 mL 70 % ethanol, and centrifuge for 10 min at $4,500\times g$ at 4°C . Remove supernatant, let pellets dry, and resuspend the PCR product into water. The PCR product is used as a template for in vitro transcription described below.

3.3 Initial In Vitro Selection

The following protocol describes a typical selection cycle. The reaction conditions should be adjusted during the selection to increase selection stringency. Conditions used during a successful selection are shown in Table 1.

3.3.1 In Vitro Transcription (IVT)

1. Mix following components on ice to a final volume of 100 μL : 10 $\times$ T7 reaction buffer, 10 μL ; 10 $\times$ NTPs mix, 10 μL ; $\alpha\text{-P}^{32}$ GTP, 1 μL ; 100 pmol DNA template; and T7 RNA polymerase, 2 μL (*see Note 1*). Incubate for 3 h at 37°C .
2. The DNA template is then degraded by addition of RQ I DNase followed by 30 min incubation at 37°C .
3. Add 25 μL 10 M ammonium acetate and 250 μL 100 % ethanol, and vortex. Centrifuge at $15,000\times g$ for 20 min at 4°C . Remove supernatant, add 500 μL 70 % ethanol, and centrifuge at $15,000\times g$ for 10 min at 4°C . Remove supernatant, open test tube, and dry pellet for a few minutes at 37°C . Resuspend the pellet in 10 μL denaturing dye.

3.3.2 Denaturing
Polyacrylamide Gel
Electrophoresis

1. Clean glass plates, spacers (1 mm), and comb (6 wells 1.5 cm each) with water and 70 % ethanol. Place spacers between two glass plates, and clamp the two sides and bottom with binder clips.
2. Prepare 9 % polyacrylamide solution by mixing 5 mL 5× TBE, 15 mL 30 % polyacrylamide solution, 25 g urea and water to a final volume of 50 mL. After dissolution of urea start polymerization with addition of 250 μ L 20 % APS and 25 μ L TEMED. Cast gel immediately. If necessary remove air bubbles by knocking gently on glass plates. Place comb and wait until gel is polymerized (30 min to 1 h). Remove comb and remove polyacrylamide rests.
3. Assemble gel on electrophoresis apparatus with aluminum plate for heat dispersion and fill reservoirs with 0.5× TBE. If needed remove air bubbles in the wells and at the bottom of the gel using a syringe.
4. Connect gel to power supply and pre-run for 30 min at 500 V (*see Note 2*). Stop power supply and rinse the wells with 0.5× TBE using a syringe with a needle to remove urea. Immediately load RNA on a well. Run the gel for approx. 1 h at 700 V until bromophenol blue is out.
5. Remove gel from apparatus and carefully separate glass plates. The gel should remain on one plate. Place the plate with the gel on wrapping film, remove the second glass plate. Place wrapping film on the other side of the gel.
6. Place the gel on a thin layer chromatography plate and illuminate with UV light (254 nm), the RNA should appear as a dark shadow. Cut out a band corresponding to the full length RNA. Place the gel piece containing RNA into a 1.5 mL tube. Add 550 μ L RNA elution solution and incubate for overnight at 37 °C (*see Note 3*).
7. To purify the RNA, add 500 μ L TE-saturated phenol, vortex strongly, and centrifuge for 5 min at 15,000×*g*. Transfer the aqueous phase to a new tube. Add 750 μ L diethylether, vortex, and centrifuge as previously. Remove the diethylether phase. Add 900 μ L 100 % ethanol. Incubate for 20 min at −20 °C and centrifuge for 20 min at 15,000×*g* at 4 °C. Remove supernatant, add 1 mL 70 % ethanol, and centrifuge for 10 min at 15,000×*g* at 4 °C. Remove supernatant, let pellets dry, and resuspend the RNA into water.
8. Quantify the RNA with a UV spectrophotometer (*see Note 4*). By following the procedure above, $\approx 1,000$ pmol (7×10^{13} molecules, eight copies for each sequence) of the RNA is obtained. The two pools (pool L and R) are mixed to prepare 2,000 pmol of RNA for the selection.

3.3.3 Initial In Vitro Selection

Scheme of the selection procedure is shown in Fig. 2.

1. Denature the prepared RNA pool described above at 80 °C for 3 min, followed by preincubation at 37 °C for 5 min.
2. Add 5× reaction buffer at 37 °C to initiate the RNA folding, and incubate at 37 °C for 10 min.
3. Add a 10× substrate RNA (S-1 or S-2 RNA) to start the ligation reaction. The final concentrations of the pool RNA, the substrate RNA, and Tris-HCl (pH 7.7) are 1 μM, 2 μM, and 30 mM, respectively. Two sets of 5'-biotinylated substrate RNAs (S-1 and S-2) and their guide sequences (types 1 and 2) are used alternatively (*see* Fig. 1 and Table 1). The S-1/type 1 set was used in rounds 1, 3, and 5, and the S-2/type 2 set was used in rounds 2 and 4 in our experiment.
4. Stop the ligation reaction by ethanol precipitation described above.
5. Capture the ligated product RNAs by incubating with streptavidin paramagnetic particles at 37 °C, and hybridize with a DNA primer complementary to their 3' region (*see* Note 5).
6. Perform reverse transcription at 42 °C with ReverTra Ace and a primer, Rv-S in rounds 1 and 4, Rv-M in rounds 2 and 5, or Rv-L in round 3, respectively (*see* Note 6).
7. Elute the resulting cDNAs by adding 150 mM KOH, followed by neutralization with 150 mM HCl (*see* Note 7).
8. Amplify the product cDNAs selectively by PCR using the primer described above together with the selective primers complementary to the sequences of the substrate RNAs (*see* Note 8).
9. Regenerate template DNA for IVT by PCR with a primer containing T7 promoter sequence together with the primer containing the template for the guide sequence of type 1 or 2.
10. (Optional) Purify the PCR product by agarose gel electrophoresis if it contains multiple bands or smear.
11. Use the resulting DNAs as templates for the next round, and repeat the whole selection cycle until catalytic species are accumulated.

3.4 Isolation of Individual Ribozymes

1. Isolate the ligated RNAs from the final round pool by using 5 % denaturing PAGE.
2. Amplify the RNAs by reverse transcription and PCR, and clone into pGEM-T vectors (*see* Note 9).
3. Determine the sequences of the individual plasmid clones by using dideoxy sequencing kit (Thermo Sequenase™ Primer Cycle Sequencing Kit) with an automated DNA sequencer (4300 DNA Analyzer).
4. Prepare the individual ribozyme RNAs by PCR, IVT, and purification by 9 % denaturing PAGE described above.

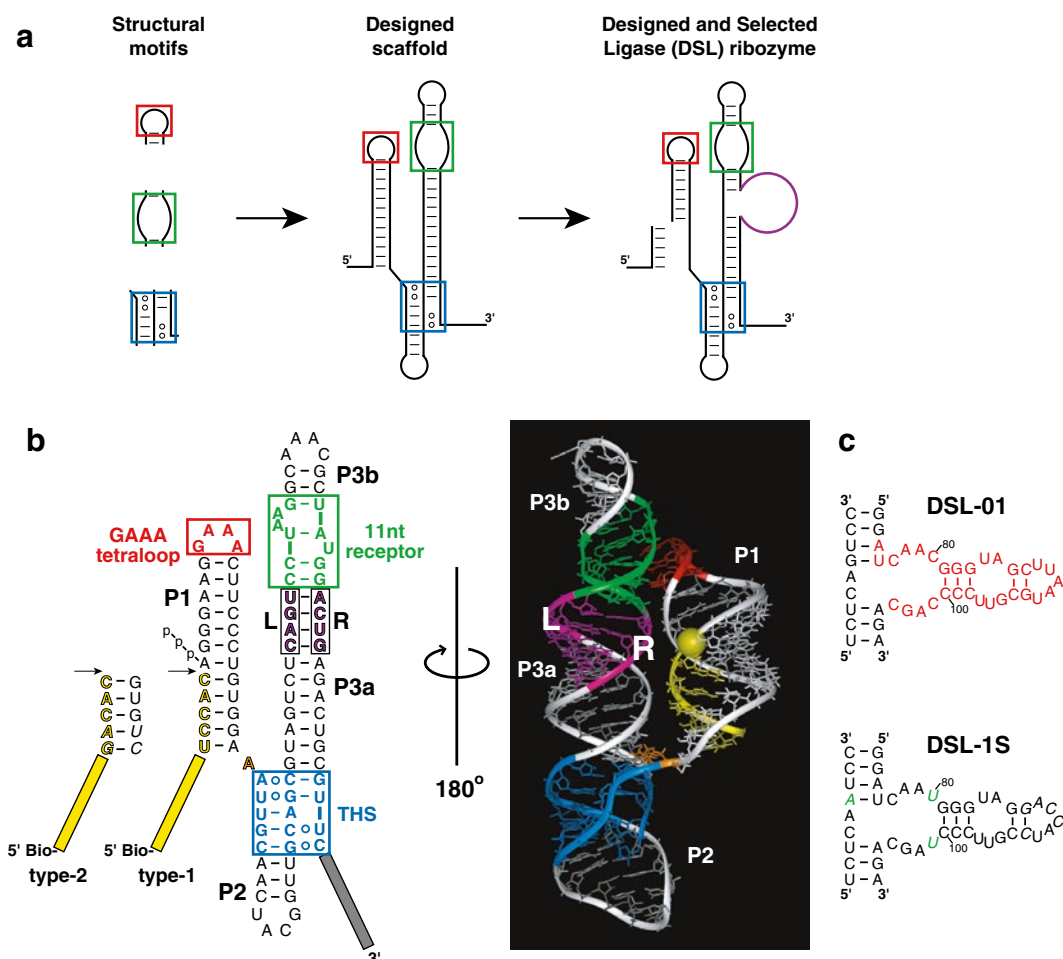


Fig. 1 Synthetic scheme and structures of DSL ligases. **(a)** From *left to right*, structural motifs, GAAA loop, 11-nt receptor, and triple-helical scaffold, used in the molecular design of the scaffold (*left*), simplified secondary structures of the scaffold (*center*), obtained the designed and selected ligase (DSL) ribozyme (*right*). A putative secondary structure of the selected catalytic module inserted at the R region in **b** is shown. GAAA loop specifically interacts with the 11 nt receptor. Nicks in the DSLs correspond to the reaction site. **(b)** Secondary (*left*) and 3D structure (*right*) of the scaffold RNA. (*Left*) The putative reaction site is indicated with an arrow. Two sets of substrate oligonucleotides and their complementary sequences (*left*, S2/Type 2; *right*, S1/Type 1) are shown. L and R indicate the regions replaced with 30 random nucleotides for constructing the libraries. Bio indicates biotin. (*Right*) A model 3D structure. Colored regions correspond to those in the secondary structure. Sphere indicates the reaction site. To show the relative position between the reaction site and L or R region without obstacles, a view from the side opposite to that shown at *left* is depicted. **(c)** Secondary structures of the selected catalytic module. (*Upper*) Proposed secondary structure of the catalytic module of DSL-01 which consists of the selected 30 nucleotides (*red*) and its adjacent regions. (*Lower*) The sequence of DSL-1S. The nucleotides responsible for improving the activity are marked with *green*. *Italic letters* correspond to the nucleotides different from those in DSL-01 (*upper*)

3.5 Ribozyme Activity Assay

1. Dissolve the uniformly ^{32}P -labeled ribozyme ligases prepared above in 35 μL of water, then denature by incubating at 80 $^{\circ}\text{C}$ for 3 min, followed by preincubation at 37 $^{\circ}\text{C}$ for 5 min.

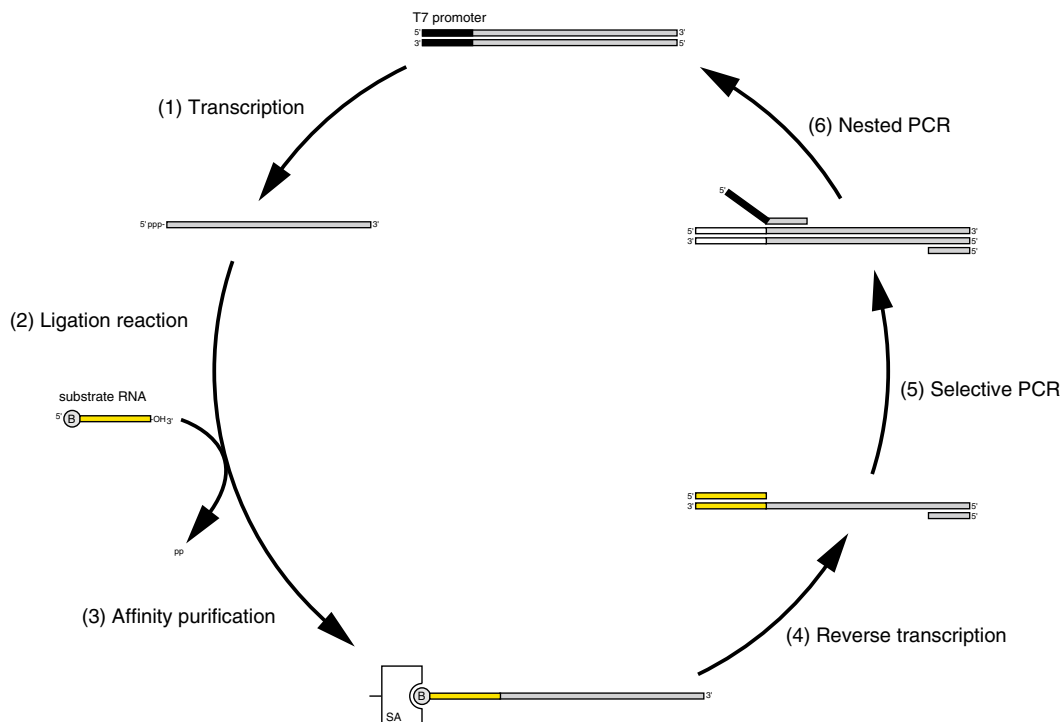


Fig. 2 Schematic representation of the in vitro selection procedure. (1) The DNA pool is transcribed using T7 RNA polymerase. (2) Ligation reaction is performed with the 5'-biotinylated substrate RNA. (3) The ligated products harboring biotin at the 5' ends are captured by incubating with streptavidin paramagnetic particles. (4) cDNA is synthesized by reverse transcription. (5) The ligated products are specifically amplified by the selective PCR with the primer complementary to the substrate. (6) T7 promoter is regenerated by the nested PCR. The resulting DNA is used for the next selection cycle

2. Initiate RNA folding by adding fivefold concentrated reaction buffer (10 μ L), then incubate at 37 $^{\circ}$ C for 10 min.
3. Add a tenfold concentrated substrate RNA (5 μ L) to start the ligation reaction. The standard conditions are as follows. Final ligase and substrate concentrations are 25 nM and 1 μ M, respectively. The reaction is performed with MgCl₂, KCl, and Tris-HCl (pH 7.7) (50, 200, and 30 mM, respectively) at 37 $^{\circ}$ C.
4. At each time point, make a 5 μ L of aliquot and treat with 5 μ L of the stop solution.
5. Analyze the samples by 5 % denaturing PAGE and quantify the gel with a Bio-Imaging Analyzer.
6. Determine the observed rate constant (k_{obs}) by fitting the data to the following equation:

$$\text{Fraction reacted} = F_a (1 - e^{-k_{\text{obs}} t}),$$

where t equals time, k_{obs} equals the observed rate constant, and F_a equals the fraction of the substrate-ribozyme in an active conformation.

3.6 Second (Doped) In Vitro Selection

3.6.1 Construction of Doped Pool

After the initial selection, the second in vitro selection experiment is carried out to isolate much higher active variants from doped (partially randomized) library. In our case, we prepared the doped pool based on the DSL-01 ribozyme which is dominant species from the initial selection (Fig. 1).

1. Construct a doped DSL-01 library by primer extension reaction with two synthetic oligonucleotides, KTL-1 dope sense and KTL-1 dope antisense. Perform the primer extension with Ex *Taq* polymerase by using 1,000 pmol of each oligonucleotide.
2. Prepare the RNA pool by IVT with the primer extension product.
3. Purify the resulting RNAs by 5 % denaturing PAGE followed by precipitation with ethanol. Amount of obtained RNA was 800 pmol.

3.6.2 Doped In Vitro Selection and Activity Assay

1. Perform the in vitro selection from the doped DSL-01 library in a similar manner to the description in Subheading 3.3.3. Selection conditions are shown in Table 1.
2. Isolate, sequence, and analyze the RNAs after the fourth round of selection by following described above. We have obtained a variant named as DSL-1S from the doped pool (Fig. 1), which is approximately tenfold active rather than the original DSL-01.

4 Notes

1. Labeling of the RNA with a fluorophore (e.g., BODIPY) is also available instead of the radioactive labeling, but it is recommended to employ the radio labeling to trace the RNA molecules effectively during the in vitro selection procedure. We can precisely monitor the fractions of RNAs which is enriched or washed away after the selection, helping a lot when we decide the selection stringency.
2. The pre-run step is important to obtain a clear result of denaturing polyacrylamide gel electrophoresis particularly when our RNA forms a stable secondary structure. If it is not enough, initial temperature of the gel before running will not be enough to unwind the RNA string, resulting in abnormal migration.
3. For the eluting out of RNA from the gel, many other protocols recommend to crash the gel. We have noticed, however, it does not facilitate the final yield of RNA at all. We propose not to crash the gel to make the following purification step easier.

4. For the quantification of RNA, many types of UV spectrophotometers are available including the NanoDrop (Thermo Scientific), but we have realized that the NanoDrop sometimes outputs an incorrect measurement. It is highly recommended to verify its accuracy by comparing with a conventional one if we plan to use it.
5. The appropriate selection stringency is very important for a successful in vitro selection experiment. Monitoring of the enrichment by radioactivity quantification is helpful to consider it. When we can see significant increase of radioactivity after washing the beads, we should apply more harsh condition for the next round to enrich more active species effectively.
6. Mainly two groups of reverse transcriptases are available; M-MLV RT (Moloney Murine Leukemia Virus Reverse Transcriptase) and AMV RT (Avian Myeloblastosis Virus Reverse Transcriptase). The M-MLV RT is more commonly used in many cases due to its low nuclease activity, but it would be also useful to try the AMV RT when our RNA is expected to form a stable secondary structure. We can increase the reaction temperature due to its high thermostability.
7. This neutralization step is important. If it is not done precisely, the residual acidic or basic pH will inhibit the following PCR reaction. We should verify in advance the mixing of the KOH and HCl solutions produces proper neutral pH by using a pH indicator.
8. It is not recommended to perform too many cycles of PCR at this step. It will generate unusual retardation of electrophoresis migration due to the randomized sequence in the DNA. We should check the PCR product outcome at each cycle by agarose gel electrophoresis, and limit the cycles not to see the retardation.
9. Other T-vector systems are also available, e.g., TOPO-TA cloning kit (Invitrogen).

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Engineering Aptazyme Switches for Conditional Gene Expression in Mammalian Cells Utilizing an In Vivo Screening Approach

Charlotte Rehm, Benedikt Klauser, and Jörg S. Hartig

Abstract

Artificial RNA switches are an emerging class of genetic controllers suitable for synthetic biology applications. Aptazymes are fusions composed of an aptamer domain and a self-cleaving ribozyme. The utilization of aptazymes for conditional gene expression displays several advantages over employing conventional transcription factor-based techniques as aptazymes require minimal genomic space, fulfill their function without the need of protein cofactors, and most importantly are reprogrammable with respect to ligand selectivity and the RNA function to be regulated. Technologies that enable the generation of aptazymes to defined input ligands are of interest for the construction of biocomputing devices and biosensing applications. In this chapter we present a method that facilitates the in vivo screening of randomized pools of aptazymes in mammalian cells.

Key words Riboswitch, Aptazyme, Ribozyme, Biosensor, Synthetic biology

1 Introduction

Synthetic genetic switches and sensors are valuable genetic controllers for synthetic biology applications, including biocomputing, metabolic engineering, and therapeutics. They are characterized by a common architecture comprised of a sensor unit, processing unit, and an actuator unit that convert environmental and cellular signals into defined outputs [1].

Synthetic riboswitches are emerging genetic controllers of gene expression consisting exclusively of RNA and execute their function in a protein-independent fashion [2]. They possess an aptamer domain as sensor that triggers a conformational change within the expression platform upon ligand binding, thereby affecting transcription, translation, or splicing of a messenger RNA.

In contrast to protein-based genetic controllers they require less genomic space. Of particular advantage is their modular architecture that facilitates reprogramming of ligand selectivity. These attributes make synthetic riboswitches powerful systems for synthetic biology applications.

Our group has developed synthetic riboswitches that undergo cleavage of the RNA backbone in a small molecule- [3, 4], temperature- [5], and trans-acting RNA-dependent [6] fashion. The trademark of the concept is to fuse the RNA aptamer to a ribozyme, yielding an aptazyme. Rational design, screening, and selection of libraries in which the connection sequence between the aptamer and ribozyme is randomized enables the identification of ligand-dependent aptazymes [7]. In recent studies aptazymes were applied to control translation of mRNA [3], tRNA maturation [8], pri-miRNA processing [9], and 16S rRNA integrity [10]. In addition, aptazymes are used in synthetic biology applications to control gene expression in mammalian cellular systems [11–13]. Mulligan and co-workers initially demonstrated that mammalian gene expression can be regulated by insertion of a hammerhead ribozyme (HHR) into either the 5′- or 3′-Untranslated region (UTR) of an mRNA [14]. Autocatalytic cleavage results in the degradation of the mRNA due to the removal of the 5′-cap or the 3′-poly-A-tail. Our group further developed this concept and reported theophylline-dependent aptazyme switches for conditional gene expression in mammalian cells [11]. Theophylline-dependent genetic switches were obtained by in vivo screening of a randomized aptazyme library. For future applications it is of major interest to engineer aptazymes with user defined ligand selectivity. In this chapter, we describe a straightforward procedure that enables the in vivo screening for functional aptazymes in mammalian cell culture. Our switches are based on the *Schistosoma mansoni* HHR (Fig. 1) [15]. Importantly, the ribozyme sequence was optimized to not contain any additional AUG codons, acting as potential translation initiation sites in the course of ribosomal 5′-UTR scanning. Randomized aptazyme sequences are inserted in the 5′-UTR of the hRluc reporter gene on the psiCHECK^(TM)-2 vector rendering *Renilla* luciferase expression ligand-dependent. Expression of firefly luciferase encoded on the same plasmid is not ribozyme-dependent and can therefore be used for normalization in a dual luciferase assay making co-transfection with a second reporter plasmid obsolete.

2 Materials

All solutions are prepared using ultrapure water and analytical grade reagents are used. Reagents are stored at room temperature unless otherwise noted.

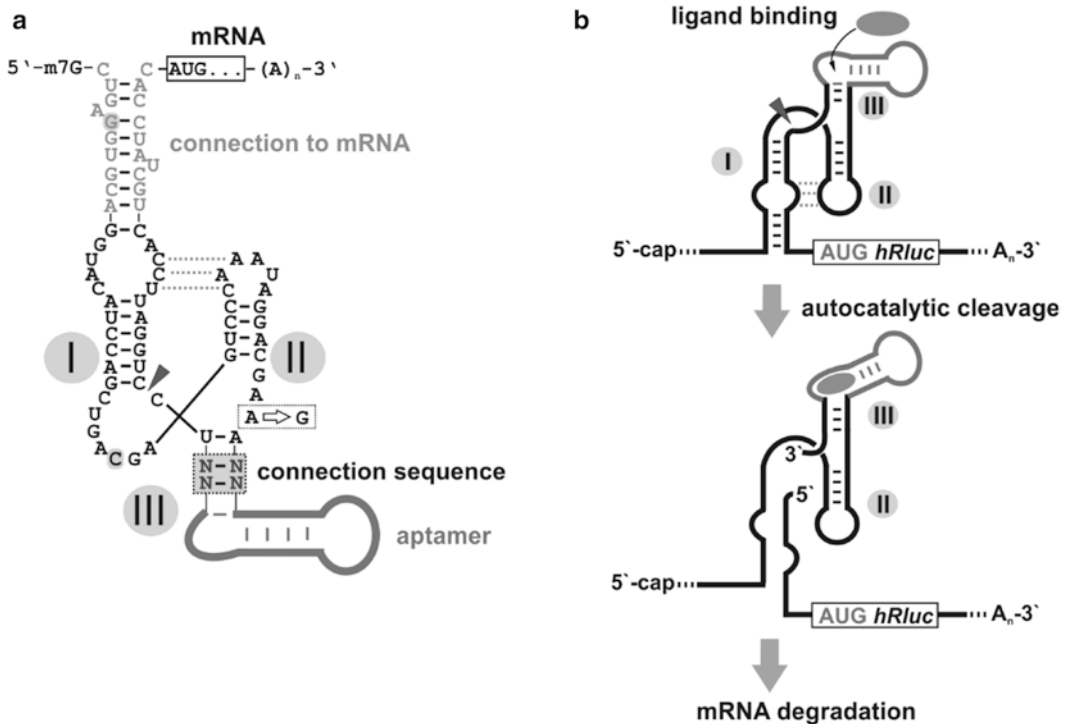


Fig. 1 Schematic illustration of genetic switches employing the hammerhead ribozyme as expression platform and proposed mechanism. **(a)** The secondary structure of the HHR and its connection to the mRNA is shown. An aptazyme library is created by attaching an aptamer domain to stem 3 of the HHR and randomization of the connecting nucleotides. The HHR is derived from *S. mansoni* and inserted via stem 1 into the 5'-UTR of a *Renilla* luciferase reporter gene. Mutated nucleotides to avoid potential alternative translation initiation sites are shaded in *grey*; tertiary interactions between stem 1 and 2 are indicated by dotted lines, and the site of autocatalytic cleavage is marked by an *arrowhead*. An A-to-G mutation within the catalytic core inactivates ribozyme catalysis. **(b)** Ligand-binding to the aptamer domain locks the ribozyme in its catalytically active conformation. Cleavage of the RNA backbone results in mRNA decay due to the removal of the 5'-cap and reduced reporter gene expression

2.1 Construction of Randomized Aptazyme Libraries

1. 10 μ M DNA oligonucleotides as primers to be used in PCR (*see* Subheading 3.1).
2. PCR template: luciferase expression vector (e.g., Rz1A_woAUG derived from psiCHECK^(TM)-2, active HHR without AUGs in 5'-UTR of Rluc (Hartig Group, University of Konstanz)) (*see* Note 1).
3. Phusion Hot Start II High-Fidelity DNA polymerase (NEB). Store at -20°C .
4. 5 $\times$ High-Fidelity buffer. Store at -20°C .
5. 100 % DMSO. Store at -20°C .
6. 2 mM dNTP Mix. Store at -20°C .
7. PCR cyclor.

8. Tabletop centrifuge.
9. 3 M Na-acetate, pH 5.2: Dissolve 20.4 g $\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$ in 50 mL H_2O . Adjust to pH 5.2 with HCl.
10. 99 % ethanol.
11. DpnI restriction enzyme and CutSmart buffer (NEB). Store at -20°C .
12. 6× agarose gel loading buffer: Dissolve 4 mL 50 mM Tris-acetate, pH 7.6, 12 mL 100 % glycerol, 2.4 mL 0.5 M EDTA, 6 mg bromophenol blue, 6 mg xylene cyanol in 20 mL, prepare aliquots of 1 mL. Buffer can be stored at -20°C for several months.
13. 0.5 M EDTA, pH 8.0: Dissolve 73.08 g EDTA and 30 g NaOH pellets in 400 mL H_2O , adjust pH 8.0 using NaOH, adjust volume to 500 mL.
14. 5× TBE buffer: Dissolve 54 g Trizma base, 27.5 g boric acid, 20 mL 0.5 M EDTA (pH 8.0) in 1 L of H_2O . If needed, adjust to pH 8.3 with concentrated HCl. Dilute to 0.5× in H_2O before use.
15. Agarose gels. For a 0.8 % (w/v) gel dissolve 0.8 g agarose in 100 mL 0.5× TBE buffer by boiling in the microwave oven. Dissolved agarose can be stored for a week in an incubator at $\geq 70^\circ\text{C}$ (*see Note 2*).
16. Ready-to-use DNA size standard (e.g., GeneRuler 1 kb DNA Ladder (Thermo Scientific)).
17. Ethidium bromide gel staining solution. Dilute 200 μg in 400 mL H_2O . Solution can be reused when kept in the dark at room temperature (*see Note 3*).
18. Destaining solution: 400 mL H_2O . Can be kept at room temperature and reused.
19. Agarose gel electrophoresis chamber and power supply.
20. Razor blade.
21. UV light table.
22. DNA Gel Extraction Kit (e.g., Gel DNA Recovery Kit (Zymo Research)).
23. Photometer to determine A_{260} .
24. Quick Ligation Kit (NEB). Store at -20°C . Thaw Quick Ligation Reaction Buffer on ice and freeze immediately after use.
25. DNA Purification Kit (e.g., DNA Clean and Concentrator-5 (Zymo Research)).

2.2 Preparation of Randomized Aptazyme Plasmid Libraries

1. Electro-competent *E. coli* (e.g., *E. coli* XL10 gold, Stratagene) (*see Note 4*). 80 μL aliquots frozen at -80°C .
2. Electroporator (e.g., Eppendorf Electroporator 2510) and electroporation cuvettes (e.g., Electroporation cuvettes, 0.1 cm (Bio-Rad)).

3. SOC medium: Dissolve 20 g tryptone, 5 g yeast extract, 0.6 g NaCl, 0.2 g KCl in 900 mL H₂O. Adjust pH to 6.8–7.0 using NaOH. Adjust volume to 960 mL and autoclave. Add 10 mL 1 M MgCl₂, 10 mL 1 M MgSO₄, and 20 mL 1 M glucose from sterile filtered stocks. Store 1 mL aliquots at –20 °C for several months.
4. 1,000× carbenicillin stock solution (100 mg/ml): Dissolve 1 g carbenicillin in 10 mL 50 % (v/v) ethanol. Solution can be stored at –20 °C for several weeks.
5. LB-Carb-agar plates: Dissolve 10 g tryptone, 5 g yeast extract, 5 g NaCl and 10 g agar-agar in 1 L H₂O and autoclave. Let cool until hand-warm, then supplement with 1 mg/L carbenicillin. Pour plates in 15 cm petri dishes. Plates can be stored at 4 °C for up to 6 weeks.
6. LB-Carb-medium: For LB (Lennox) dissolve 10 g tryptone, 5 g yeast extract, 5 g NaCl in 1 L H₂O, adjust to pH 7.0, if necessary, and autoclave. Supplement with 1 mg/L carbenicillin before use. Can be stored at 4 °C for up to 4 weeks.
7. 96 deep-well plate and 96-well plate incubator (e.g., Heidolph Inkubator 1000 and Titramax 1000).
8. Toothpicks sterilized by autoclaving.
9. Air-permeable adhesive seals for 96-well plates.
10. Kit for plasmid isolation in the 96-well format, e.g., NucleoSpin Multi-96 Plus Plasmid Kit (Macherey Nagel).

2.3 Mammalian Cell Culture and Screening

1. Mammalian cell lines, e.g., HEK293 cells (ATCC: CRL-1573) or HeLa229 (ATCC: CCL-2.1).
2. CO₂-humidified incubator.
3. Sterile bench.
4. 75 cm² cell culture flasks.
5. Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen).
6. DMEM complete medium: DMEM supplemented with 10 % heat-inactivated fetal calve serum (e.g., from PAA) and 100 U/mL penicillin and 100 µg/mL streptomycin (e.g., from GIBCO).
7. 10× PBS: Dissolve 80.06 g NaCl, 2.01 g KCl, 178.01 g Na₂PO₄·2H₂O, 2.72 g KH₂PO₄ in 950 mL H₂O, adjust pH 7.4 with NaOH, adjust volume to 1 L with H₂O and autoclave. Can be kept at 4 °C for several weeks.
8. 0.25 % trypsin–EDTA solution (e.g., from SIGMA).
9. Hemocytometer, 0.5 % (w/v) trypan blue in 1× PBS, and bright field microscope for cell number determination.
10. Water bath.
11. 50 mL conical centrifuge tubes.

12. Centrifuge equipped for 50 mL tubes, e.g., eppendorf centrifuge 5810R.
13. 50 mL reagent reservoirs for multichannel pipettes.
14. 5–50 μ L and 20–200 μ L multichannel pipettes.
15. 96-well plates for cell culture (Becton Dickinson).
16. Transfection reagent, e.g., Lipofectamine 2000 (Invitrogen).
17. Luciferase assay kit, Dual-Luciferase[®] Reporter Assay Kit (Promega).
18. 96-well plates with conical bottom.
19. 96-well half-area plates, black for luminescence measurement, e.g., Corning costar 3694.
20. Microplate reader/luminometer, e.g., Tecan M200.

3 Methods

3.1 Construction of Randomized Aptazyme Libraries

A plasmid pool of aptazyme mutants is created by incorporation of new aptamer sequences into the HHR using whole plasmid PCR as done for site-directed mutagenesis (Fig. 2). In order to randomize the connection sequence between aptamer and HHR primers carrying unbiased random positions generated during solid phase DNA synthesis using a 1:1:1:1 mixtures of nucleoside phosphoramidites are used. 5'-Phosphorylation of one primer is required for self-ligation of the PCR product. To improve the quality of the library, we recommend ordering primers ≥ 40 nts with HPLC purification or to purify primers ≥ 70 nts by denaturing PAGE, as for example described by Sambrook and Russel (Preparation of Denaturing Polyacrylamide Gels; Purification of Synthetic Oligonucleotides by Polyacrylamide Gel Electrophoresis).

1. Prepare the reaction mixture as detailed below, the reaction can be scaled up if necessary. Split the mixture into PCR tubes, we recommend using 20 μ L per tube (*see* Note 5).

Starting concentration	Material	Volume	Final concentration
5×	HF Buffer	12	1×
2 mM	dNTP mix	6	200 μ M
10 μ M	Forward primer	3	500 nM
10 μ M	Reverse primer	3	500 nM
10 ng/ μ L	Template	1.2	12 ng
100 % (v/v)	DMSO	1.8	3 % (v/v)
2 U/ μ L	Phusion Hot Start II DNA polymerase	0.6	1.2 U

(continued)

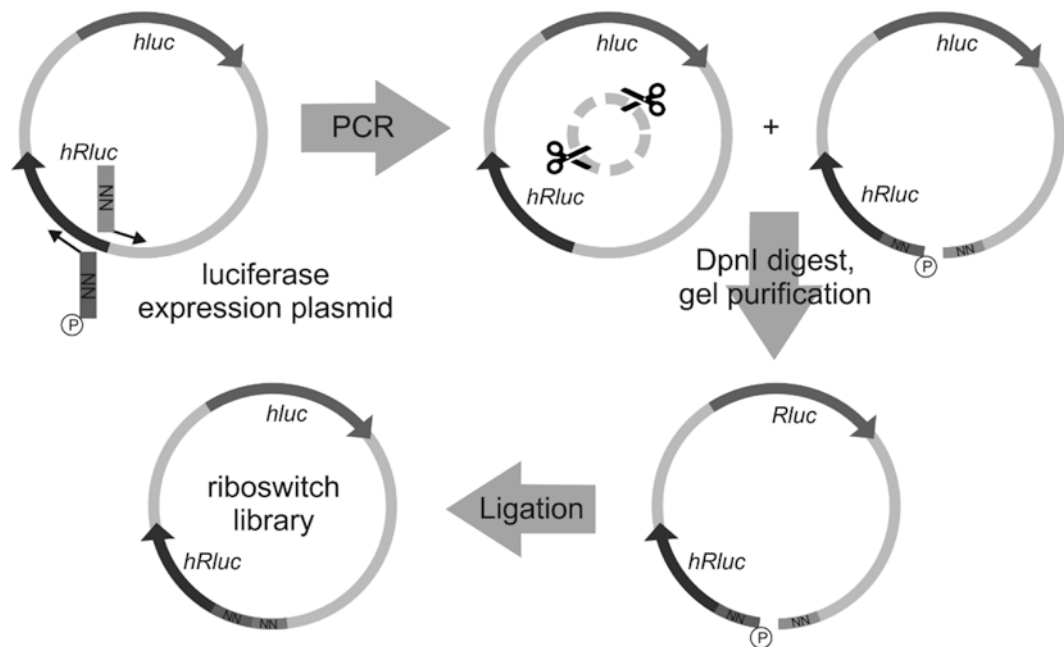


Fig. 2 Construction of randomized aptazyme libraries. The aptazyme is incorporated in the 5'-UTR of the *hRluc* reporter gene on psi-CHECK2 to screen for ligand-dependent expression of *Renilla* luciferase in dual luciferase assay. Expression of firefly luciferase from the *hLuc* gene present on the same plasmid is used for normalization. Whole plasmid PCR is used for incorporation of randomized aptazyme sequences using primers carrying randomized nucleotide positions that will constitute the connecting sequence. DpnI digest removes the methylated template plasmid. Ligation of the purified PCR product yields pool of plasmids carrying randomized aptazymes

(continued)

Starting concentration	Material	Volume	Final concentration
	H ₂ O	32.4	
	Total	60	

2. The following conditions are applied for the PCR with Phusion Hot Start II High-Fidelity DNA polymerase.

Step	Stage	Temperature (°C)	Time	Go to step	Repeat
1	Initial denaturation	98	30 s		
2	Denaturation	98	10 s		
3	Annealing	60	30 s		
4	Extension	72	15–30 s/kb	2	24
5	Final extension	72	7 min		
6	Cooling	4	Pause		

3. Pool the PCR reaction mix into one 1.5 mL reaction tube. For ethanol precipitation add 1/10 volume of 3 M Na-acetate, pH 5.2, followed by 3 volumes 99 % ethanol and mix by inverting.
4. Place samples at -80°C for 20 min or -20°C for a minimum of 2 h or overnight.
5. Centrifuge samples in a tabletop centrifuge at $12,000\times g$ for 15 min at room temperature. Discard supernatant, wash once with 70 % ethanol. Resuspend the pellet in 44 μL H_2O .
6. For digestion of the methylated template vector add 5 μL CutSmart buffer and 1 μL DpnI. Mix thoroughly. Incubate at 37°C for 60 min followed by 10 min incubation at 80°C for heat inactivation of the enzyme.
7. In the meantime, pour a 0.8 % (w/v) TBE-agarose gel using a comb that will provide a large pocket for the PCR product and a small pocket for the size standard. Once the gel has solidified, mix the sample with 10 μL of 6 $\times$ agarose gel loading buffer. Load the sample in the long well and load 2.5 μL DNA size standard to run alongside. Run the gel at 10 V/cm for 75–90 min.
8. Transfer the gel into ethidium bromide staining solution for 10 min, to improve contrast the gel is transferred to destaining solution for 10 min. As exposure to UV light may introduce mutations to the DNA, only part of the sample is visualized over a UV light table. Cut the gel vertically excising the marker and a small part of the band with your sample. On the UV light table excise the correctly sized band.
9. Piece both parts of the gel back together and using the incision as a marker excise the corresponding band in the gel that has not been exposed to UV light. Purify the DNA fragment using a DNA gel extraction kit according to manufacturer's protocol.
10. Determine the DNA concentration by measuring A_{260} .
11. For ligation of the PCR product combine 50 ng of the purified PCR product with 5 μL 2 $\times$ Quick Ligation Reaction buffer and 1 μL Quick Ligase in a total volume of 10 μL . Incubate at 25°C for 15 min.
12. Immediately after ligation purify the DNA sample by using a DNA purification kit according to the manufacturer's instructions to remove the ligation buffer for better transformation efficiency. Elute ligated DNA using 6 μL H_2O .

3.2 Preparation of Randomized Aptazyme Plasmid Libraries

A plasmid library of aptazyme mutants carrying randomized connecting sequences is obtained by transformation of the randomized plasmid pool into *E. coli* (Fig. 3). Single clone colonies are picked and isolated in the 96-well format.

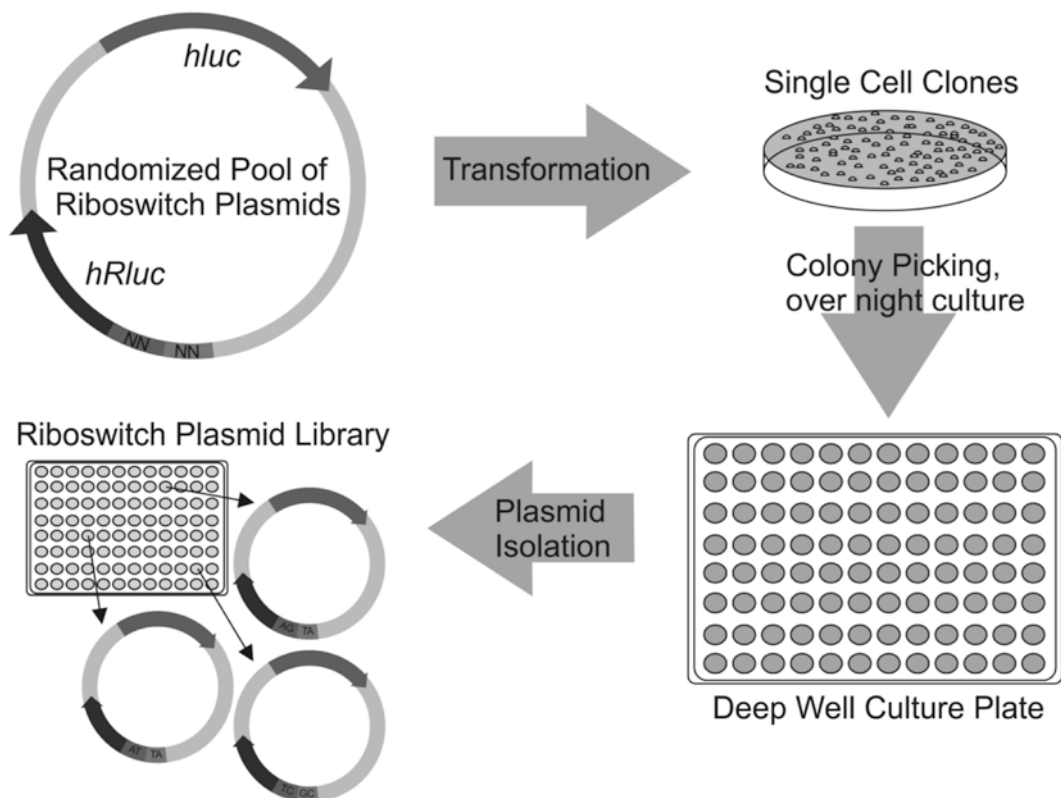


Fig. 3 Preparation of randomized aptazyme plasmid libraries. *E. coli* are transformed with the pool of randomized riboswitch plasmids. Single cell colonies are picked for overnight cultures in 96 deep-well plates. Plasmid preparation is carried out in the 96-well format yielding a plasmid library to be used for screening

1. Thaw 80 μ L aliquot of electro-competent *E. coli* on ice for 15 min. In the meantime, thaw 1 mL aliquot of SOC medium and preheat to 37 $^{\circ}$ C, place LB-Carb-agar plates on 37 $^{\circ}$ C and chill one electroporation cuvette on ice along with the purified, ligated DNA sample from the previous step.
2. Add 1.0 μ L of the purified sample to 80 μ L competent cells and carefully mix on ice. Transfer the cells into the prechilled electroporation cuvette and transform by electroporation with a pulse of 1,800 V. Immediately resuspend the cells in pre-warmed 1 mL SOC medium and transfer to a fresh 1.5 mL reaction tube. Incubate for 1 h at 37 $^{\circ}$ C. Plate the transformed cells on pre-warmed LB-agar plates and incubate plates overnight at 37 $^{\circ}$ C (*see Note 6*).
3. Prepare 96 deep-well plates with 1 mL LB-medium supplemented with carbenicillin to be used as culture plates. One may add control cultures to be directly included in the library. Using sterilized toothpicks, pick single colonies from the pool of mutants (*see Note 7*). Seal plates with air-permeable adhesive cover and incubate at 37 $^{\circ}$ C overnight in a 96-well plate incubator.

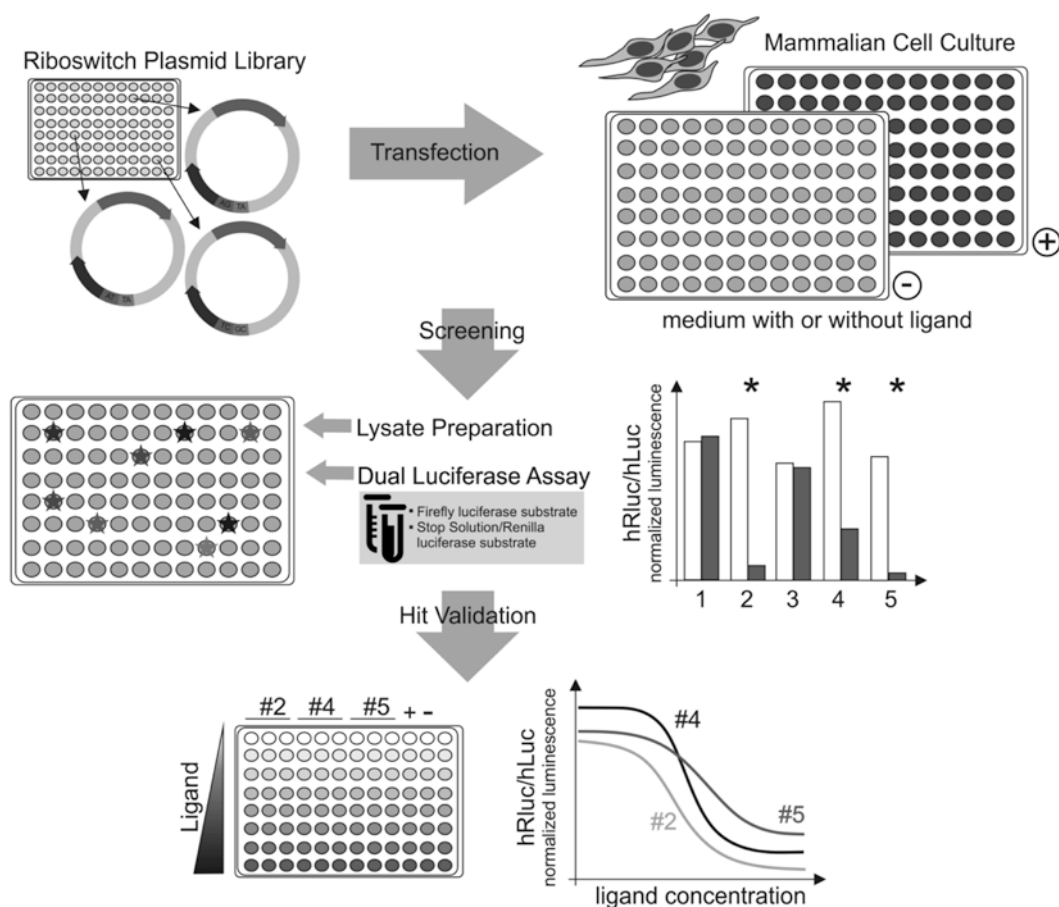


Fig. 4 Screening for functional aptazymes in mammalian cells. The plasmid library is transfected into mammalian cells by standard transfection methods. Cells are then incubated in the presence and absence of aptazyme controlling ligand. Cell lysates are prepared and assayed using a dual luciferase assay and hits are identified by comparing normalized luminescence in absence and presence of the ligand. Active aptazymes are then further characterized

- Plasmids are isolated using a Plasmid isolation Kit of the 96-well format according to the manufacturer's protocol. For easier handling during transfection plasmid concentrations are adjusted to 50 ng/ μ L.

3.3 Screening for Functional Aptazymes in Mammalian Cells

Plasmids carrying randomized aptazymes are transfected into mammalian cells and incubated with or without the respective aptazyme ligand. Screening for allosteric aptazymes is carried out in the 96-well format using a dual luciferase assay (Fig. 4). *Renilla* luciferase expression is dependent on aptazyme activity, while expression of firefly luciferase is not ribozyme-dependent and will be used for normalization; co-transfection with a second plasmid expressing a reporter is therefore not required. All procedures with

mammalian cells have to be carried out in a sterile atmosphere with sterilized equipment. Use of a multichannel pipette for working in the 96-well format is advised.

1. HEK293 cells are maintained in 75 cm² culture dishes in DMEM full medium at 37 °C in a humidified incubator at 5 % CO₂ atmosphere and passaged regularly at 70–80 % confluency.
2. On the day before transfection cells from one 75 cm² plate are washed with 10 mL pre-warmed 1× PBS. 1 mL 1× trypsin–EDTA is added and the plate is incubated at 37 °C for 3 min until the cells detach from the flask. The cells are resuspended in 9 mL of pre-warmed DMEM full medium and transferred to a 50 mL tube. A sample is taken for cell number determination and vital staining with trypan blue with a hemocytometer, the remaining cells are collected by centrifugation 200×*g* for 5 min to remove trypsin containing medium. After resuspension in fresh complete medium cells are plated at 15,000 cells per 100 µL in 96-well plates and incubated at 37 °C in a humidified incubator at 5 % CO₂ atmosphere over night.
3. On the next day cells are transiently transfected with 50 ng plasmid using the Lipofectamine 2000 reagent according to manufacturer's protocol. Briefly, in a 96-well plate transfection mixtures are prepared using plasmid from the plasmid library, Lipofectamine 2000 reagent and DMEM without serum and antibiotics. Sufficient material should be prepared to allow transfection of cells to be incubated with and without the ligand at least in duplicate. The mixture is added dropwise to the cells. Cells are then incubated at 37 °C for 6 h (*see Note 8*).
4. 6 h after transfection, the medium is carefully exchanged with fresh DMEM full medium supplemented with the aptazyme ligand or without. The ligand stock solution should be prepared in sterile 1× PBS. Cells are incubated with or without the ligand for 24 h (*see Note 9*).
5. To quantify the change in hRLuc gene expression, luciferase activity is detected using the Dual Luciferase Assay Kit according to the manufacturer's instructions. Briefly, cells are carefully washed once with sterile 1× PBS and then lysed using 1× Passive Lysis Buffer by shaking at room temperature. Cell lysates are transferred to a fresh 96-well with conical bottom, cell lysates can be kept on ice for some hours or be used immediately. In a black 96-well half-area plate mix cell lysate with Luciferase Assay Reagent II measure the luciferase activity of Firefly luciferase (hluc) immediately in the luminometer. Stop&Glo Reagent is added and Renilla Luciferase (hRLuc) activity is recorded immediately.

6. To identify potential riboswitches, luminescence of hRLuc is divided by those of hluc to obtain hRLuc/hluc after background correction.
7. Plasmids of respective hits are retrieved from the plasmid library and sent for sequencing.

4 Notes

1. Other ribozyme sequences can be used for the construction of new aptazymes, however alternative start codons need to be removed by site directed mutagenesis, conservation of bases in the catalytic core need to be considered in the process.
2. For a good separation and resolution of 5–10 kb PCR products, 0.8 % (w/v) agarose gels are sufficient. Higher percentages of agarose (e.g., 1.5 % (w/v)) can offer better resolution for smaller products.
3. Ethidium bromide is toxic and mutagenic. Wear nitrile gloves while working with it.
4. Other electro-competent bacteria can be used, we recommend using a strain with a high transformation phenotype for the creation of plasmid libraries. For the preparation of electro-competent *E. coli* a detailed protocol can be found at www.eppendorf.com.
5. Alternative to Phusion Hot Start polymerase, other polymerases can be used. The protocol then needs to be adapted to the appropriate buffer and conditions as required by manufacturer's protocol. Use of a high fidelity polymerase is advisable. Primers should be designed to exhibit an annealing temperature of 60–66 °C, however we suggest that a gradient PCR is carried out to determine the optimal annealing temperature. For difficult templates GC buffer or DMSO concentrations up to 8 % can be used.
6. Dilutions of the transformed cells are plated in order to yield single colonies. Dilutions will have to be determined experimentally as they depend on the transformation efficiency of the electro-competent cells used. A number of clones should be picked for plasmid isolation to check integrity of the library and correct insertion of the aptazyme sequence before plasmid library preparation in the 96-well format.
7. Pool coverage can be calculated by obtaining the oversampling factor (O_f) which is calculated as $O_f = \frac{T}{V} = -\ln(1 - P_i)$ where T is the number of clones actually screened, V the maximum number of different clones of a randomized sequence (sequence space) and P_i the probability that a particular sequence occurs

in the library [16]. High pool coverage is only achieved when the theoretical sequence space is oversampled multiple times.

8. As controls one can use cells transfected with a plasmid expressing Renilla luciferase with an inactive ribozyme carrying a A to G point mutation in the catalytic core (Fig. 1) as positive control and the template plasmid Rz1A_woAUG as negative control. Cells that have undergone mock transfection can be used to determine background values of luminescence.
9. Alternatively, the same volume of medium containing 2× the normal concentration of serum and antibiotics and 2× the concentration of the aptazyme ligand may be added without removing the transfection mixture. However, if toxicity is observed after transfection, the medium should be replaced with fresh complete medium. Media of different composition than DMEM will have to be considered when screening with naturally occurring ligands. Membrane permeability of the ligand and stability in the medium also have to be taken into account. When using new ligands or media we recommend to determine cell viability, e.g., in a colorimetric assay using resazurin, to determine potential toxicity of a ligand prior to screening.

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Aptazyme-Based Riboswitches and Logic Gates in Mammalian Cells

Yoko Nomura and Yohei Yokobayashi

Abstract

This chapter describes a screening strategy to engineer synthetic riboswitches that can chemically regulate gene expression in mammalian cells. Riboswitch libraries are constructed by randomizing the key nucleotides that couple the molecular recognition function of an aptamer with the self-cleavage activity of a ribozyme. The allosteric ribozyme (aptazyme) candidates are cloned in the 3' untranslated region (UTR) of a reporter gene mRNA. The plasmid-encoded riboswitch candidates are transfected into a mammalian cell line to screen for the desired riboswitch function. Furthermore, multiple aptazymes can be cloned into the 3' UTR of a desired gene to obtain a logic gate response to multiple chemical signals. This screening strategy complements other methods to engineer robust mammalian riboswitches to control gene expression.

Key words Riboswitch, Aptamer, Ribozyme, Aptazyme, Gene regulation, RNA engineering

1 Introduction

Chemical regulation of gene expression using RNA aptamers is an attractive strategy because of the versatility of in vitro selected RNA aptamers to recognize a wide variety of molecules with respectable affinity and specificity [1]. In mammalian cells, it has been demonstrated that embedding an aptazyme (allosteric self-cleaving ribozyme whose activity is engineered to be regulated by an aptamer ligand) in the 3' untranslated region (UTR) of a transgene mRNA allows chemical regulation of gene expression [2–4]. Ribozyme-mediated cleavage in the 3' UTR is expected to result in mRNA degradation and translation inhibition (Fig. 1a). Therefore, once established, a ligand-responsive aptazyme can be embedded in the 3' UTR of any mRNA to achieve chemical regulation of gene expression.

In this chapter, we describe a strategy to engineer aptazymes that function in the context of 3' UTR in mammalian mRNAs. While there are many strategies and platforms to engineer aptazymes

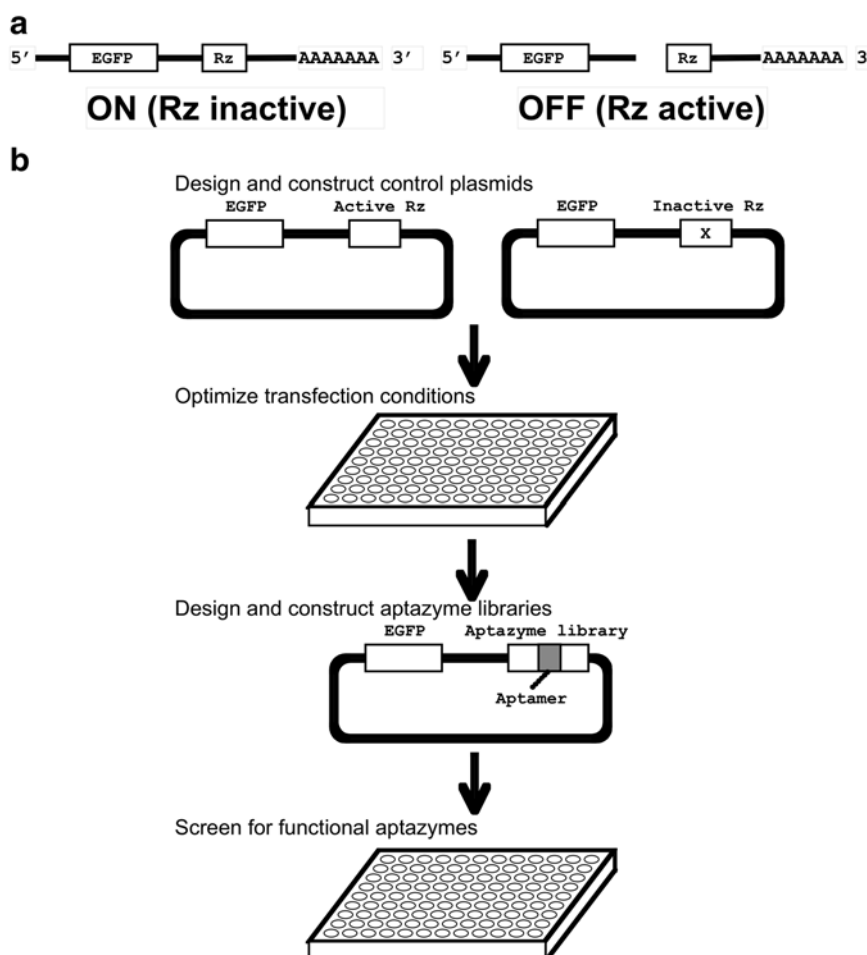


Fig. 1 Ribozyme-mediated gene regulation. **(a)** A ribozyme is embedded in the 3' UTR of a reporter gene (EGFP) mRNA. An active ribozyme cleaves the mRNA resulting in repressed EGFP expression while an inactive ribozyme allows EGFP to be expressed. **(b)** Outline of the aptazyme-based riboswitch screening strategy

in vitro and in living cells [5–8], we found that aptazymes obtained in one context do not always function as expected when applied in another context. Consequently, we established a medium-throughput screening protocol to screen several hundred aptazyme mutants directly in mammalian cell culture (Fig. 1b). First, activity of the parent ribozyme to be used must be confirmed in the context of the mammalian 3' UTR by constructing control plasmids that incorporate either an active or an inactive ribozyme downstream of a reporter gene (EGFP). These plasmids are transfected into a mammalian cell line to confirm the expected activity as well as to optimize the transfection conditions. Subsequently, aptazyme libraries are designed and constructed, and screened for the desired gene regulatory function in transfected cells.

While the strategy is somewhat labor intensive, we have discovered efficient aptazymes that function well in mammalian cells that require no further adaptation to the host cell [3]. Furthermore, multiple aptazymes can be embedded in tandem to achieve multi-input logic gate response [3, 9].

2 Materials

2.1 Cell Culture

Reagents and Cell Line

1. Fetal bovine serum (FBS).
2. Dulbecco's Modified Eagle Medium (DMEM) containing glucose and L-glutamine, without sodium pyruvate.
3. Phosphate buffered saline (PBS).
4. Trypsin-EDTA solution: 0.05 % trypsin, 0.53 mM EDTA.
5. Antibiotic antimycotic solution, 100×: 10,000 IU penicillin, 10 mg/mL streptomycin, 25 µg/mL amphotericin B.
6. HEK 293 cell line (ATCC).

2.2 Equipment

1. CO₂ incubator.
2. Inverted microscope.
3. Microplate reader (for cell fluorescence assay).
4. NanoDrop or a UV spectrophotometer for measuring plasmid DNA concentration.

2.3 Plasmids and Primers

1. pEGFP-N1 (Clontech), or a reporter gene expression plasmid of choice.
2. pCMV-mCherry [3], or a reporter gene expression plasmid for transfection control.
3. pUC19 or other inactive plasmids for adjusting DNA–transfection reagent ratio.
4. An appropriate sequencing primer to sequence the aptazyme clones.

2.4 Other Reagents and Supplies

1. Polyfect (QIAGEN) or other transfection reagents.
2. Aptamer ligand (e.g., theophylline).
3. LB medium and LB agar plates.
4. Competent *Escherichia coli* cells (e.g., TOP10).
5. Plasmid miniprep kit.
6. Other reagents and supplies that are necessary for plasmid library construction.

based on the available structural and functional information regarding the parent ribozyme to be used. An example for the HDV ribozyme is shown in Fig. 2.

2. Decide the positions of the bases to be randomized. Typically, these bases comprise the junction between the aptamer and the ribozyme. The number of randomized bases should be kept to 4–5 bases at most, to ensure that a sizable fraction of the possible mutants can be screened.
3. The library construction strategy depends on the positions of the randomized bases and the size of the aptamer. Here, several representative strategies are described briefly. If the library contains randomized bases in one contiguous (or very close) region, the best strategy is to first clone the aptamer and generate the library by whole-plasmid PCR (*see Note 1*) using primers with degenerate bases at the 5' end (Fig. 3a). If the library contains randomized bases in two distinct regions (e.g., 5' and 3' ends of an aptamer), Gibson assembly (*see Note 2*) method could be a suitable option (Fig. 3b). Alternatively, whole-plasmid PCR (*see Note 1*) using primers with degenerate bases and the aptamer sequence could be attempted if the aptamer size is relatively small (Fig. 3c).
4. Transform the plasmid library into a suitable competent *E. coli* cells (e.g., TOP10) and spread on LB agar plates. Incubate the plates at 37 °C overnight.
5. Pick 100–300 colonies and start overnight cultures (1 mL LB) in deep 96-well plates.
6. Isolate the plasmids from the overnight cultures using a plasmid miniprep kit.

3.3 Transfection of Aptazyme Library into HEK 293 Cells (See Note 3)

1. Maintain HEK293 cells in a 5 % CO₂ humidified incubator at 37 °C in DMEM supplemented with 10 % FBS and 1× antibiotic-antimycotic.
2. One day before transfection, trypsinize and dilute the HEK293 cells with fresh complete medium, and seed 2.4×10^4 cells/well (~100 µL) onto 96-well tissue culture plates.
3. Transfect 50 ng of an EGFP-aptazyme plasmid, 10 ng of pCMV-mCherry, and 50 ng of pUC19 using 1 µL of PolyFect reagent (QIAGEN) per well. Similarly transfect the appropriate controls (active and inactive ribozymes). Several wells should also be reserved for untransfected cells to be used as background wells.
4. After 3.5 h incubation, remove the medium and replace with 100 µL fresh complete medium containing appropriate concentrations of theophylline or an appropriate aptamer ligand (*see Note 4*).
5. Incubate the cells for additional 18 hr before EGFP assay (Subheading 3.4).

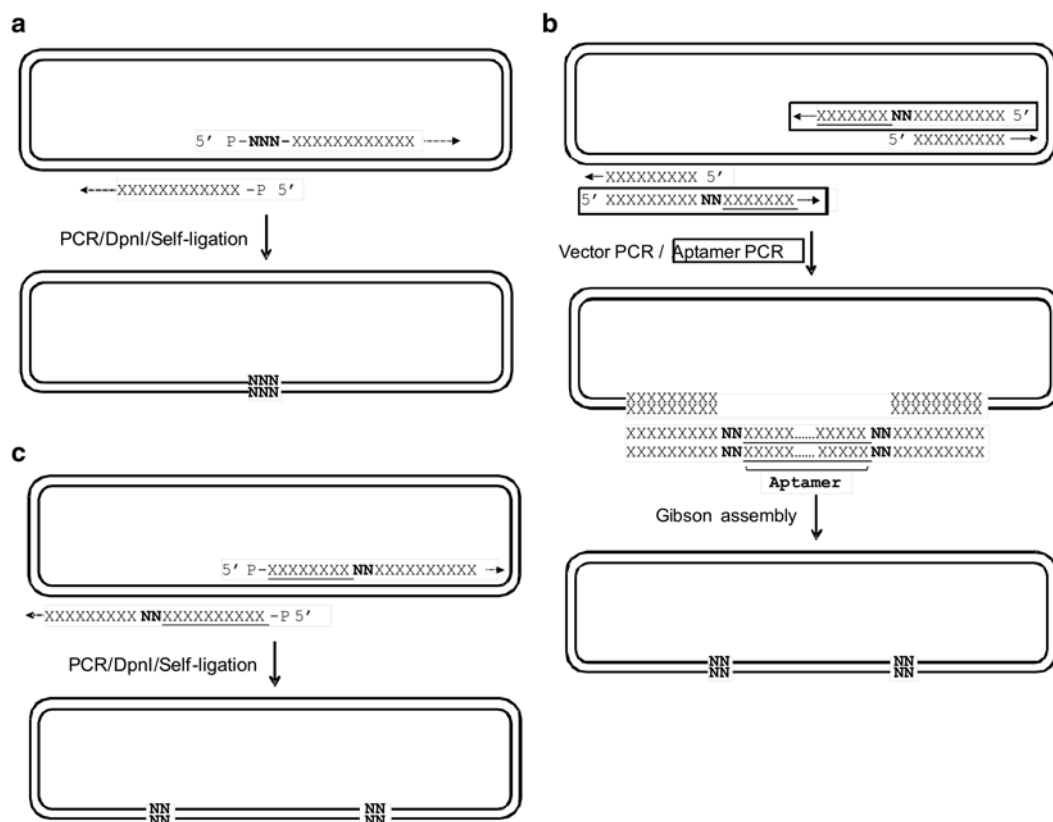


Fig. 3 Strategies for plasmid library construction. **(a)** The primers are 5' phosphorylated and one of them contains degenerate bases (N) at its 5' terminus. After whole-plasmid PCR, the template plasmid is digested by Dpn I and the PCR product is self-ligated using T4 DNA ligase to obtain the plasmid library. **(b)** Two PCR reactions are performed. First, the plasmid vector excluding the aptamer sequence is PCR amplified using an appropriate pair of primers. Second, the aptamer sequence (underlined) is PCR amplified by the primers (boxed) that add randomized bases and ~20 base overlap sequences for Gibson assembly. The two PCR products are fused by the Gibson assembly method. **(c)** The 5' phosphorylated primers include the aptamer sequence (underlined) divided between the two primers, followed by the randomized bases. The primers are used to perform whole-plasmid PCR, treated with Dpn I, and self-ligated to obtain the plasmid library. Because the primers must contain the entire aptamer sequence, this strategy is applicable to small aptamers (up to ~30 bases)

3.4 EGFP Assay

1. Replace the medium with PBS (150 μ L per well) and incubate at 37 $^{\circ}$ C until measurement.
2. Measure the fluorescence intensities of EGFP (484 nm excitation/510 nm emission) and mCherry (587 nm excitation/610 nm emission) using a microplate reader.
3. Subtract each raw fluorescence value with the background obtained from the untransfected cells.
4. For each well, normalize the EGFP fluorescence by the mCherry intensity ([EGFP fluorescence]/[mCherry fluorescence]) to

account for variations in transfection efficiency. The values should be further normalized by the cells transfected with the inactivated ribozyme control.

5. Identify aptazyme clones that display ligand-responsive normalized EGFP expression (*see* **Note 5**).

3.5 Sequence Analysis

1. Sequence the verified aptazyme clones.
2. If further improvements in aptazyme function are desired, examine the discovered sequences and design a new set of libraries, for example, by keeping some consensus bases and randomizing the previously fixed bases.

3.6 Construction of Logic Gates

1. Riboswitches that respond like Boolean logic gates may be obtained by embedding multiple aptazymes in tandem in the 3' UTR [3, 9].
2. When cloning two or more aptazymes in the 3' UTR, factors such as the order of the aptazymes and the sequence between the aptazymes may affect the quantitative characteristics of the logic gates. As such, some empirical optimization may be necessary to achieve expected characteristics.

4 Notes

1. For example, Q5 Site-Directed Mutagenesis Kit (New England Biolabs).
2. Gibson Assembly Master Mix (New England Biolabs).
3. The transfection protocol should be optimized for the specific cell line, aptamer ligand, and the reporter gene used in the experiment, using appropriate controls (Subheading 3.1). This section is presented as an example.
4. Timing of aptamer ligand addition should be optimized to minimize toxicity which can depend on both the ligand and the transfection reagent.
5. Even if no functional aptazymes are found, the gene expression data may provide useful information for designing the next round of libraries. For example, if majority of the mutants exhibit high fluorescence comparable to that of the inactive ribozyme control, it suggests that most of the mutants are too unstable to maintain the ribozyme structure, or an alternative structure that inactivates the ribozyme is too stable. Conversely, if majority of the mutants exhibit low fluorescence comparable to that of the active ribozyme control, it suggests that the mutants are stable enough to form active ribozyme structure in the absence of the aptamer ligand.

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Chapter 13

Design and Characterization of Topological Small RNAs

Jack Hassall*, Paul MacDonald*, Teresa Cordero,
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Abstract

RNA can self-assemble into complex structures through base pairing, as well as encode information and bind with proteins to induce enzymatic activity. Furthermore, RNA can possess intrinsic enzymatic-like (ribozymatic) activity, a property that, if necessary, can be activated only upon the binding of a small molecule or another RNA (as is the case in aptazymes). As such, RNA could be of use in nanotechnology as a programmable polymer capable of self-assembling into complex topological structures. In this chapter we describe a method for designing advanced topological structures using self-circulating RNA, exemplified by three tiers of topologically manipulated self-assembling synthetic RNA systems. The first tier of topological manipulation, the RNA knot is a physically locked structure, formed by circularizing one monomer of knotted single-stranded RNA left with loose ends (an “open” knot). The second tier, a two interlocking ring system, is made by interlocking two circular RNA components: a circular RNA target, and an RNA lasso designed to intercalate the target before circularizing. The third tier naturally extends this system into a string of topologically locked circular RNA molecules (an RNA chain). We detail the methodology used for designing such topologically complex RNAs, including computational predictions of secondary structure, and where appropriate, RNA-RNA interactions, illustrated by examples. We then describe the experimental methods used for characterizing such structures, and provide sequences of building blocks that can be used for topological manipulation of RNA.

Key words Topological manipulation, RNA lasso, Permuted intron–exon ribozyme, RNA knot, Interlocking RNA

1 Introduction

RNA is a powerful construction tool in nanotechnology for enabling the production of flexible self-assembling structures. Various strategies have been used for their creation as reviewed by Gao [1]. One strategy is to employ secondary structure prediction software to create self assembling RNA of a defined shape for use as building blocks in more complex architectures. Another is to use natural sequences capable of ribozymatic activity. Since ribozymes usually

*Authors contributed equally to this work.

rely on tertiary structure interactions for activity, they cannot be designed de novo using current technology. However, they can be incorporated within larger designs, permitting the creation of custom RNA architectures. The aim of this chapter is to describe computational design techniques, developed with homage to methods established in the literature [2–6], which could be used to build topologically linked RNAs using ribozymes. We consider circular RNA as our building blocks, since they are more stable than their linear counterparts [7], and have previously been successfully utilized in self-assembling systems (e.g., as riboregulators [8]). The complex architectures designed here emerge from the topological manipulation of structurally simpler circular RNA molecules. Three tiers of topological manipulation are presented, from the knotting of a single stranded RNA to the production of a chain of multiple topologically interlocked RNA molecules. We provide insight into the sophisticated structures and scaffolds one can create through current RNA modelling techniques and delve into the methods used for the detection and characterization of these structures.

The first tier of topological manipulation is seen in the self-catalytic knotting of a single RNA strand. Knots are ubiquitous in their nature; as one can observe in an inexplicably tangled set of headphones, or by glancing down at their own laced shoes. Such knots can be considered “open,” as their ends remain loose and unconnected. The sites at which each strand crosses or overlaps itself are referred to as crossing points, and whether a strand crosses over itself or under itself at each crossing point, and how many crossing points a knot has, defines that knot. The topological knots designed here are none too dissimilar; differing in that the ends are joined together so that they cannot be undone. One can achieve this by selecting previously found “open knots” in the literature [9]—or discovering more through analysis the 3D structure of known pseudoknots—and then circularizing them (Fig. 1). In 1992, Putturaju and Been developed the Permuted Intron–Exon (PIE) method to circularize a given sequence of RNA and theoretically any Group I intron can be used to achieve this (Fig. 2) [10, 11]. A particular region of Group I introns has been shown to be peripheral (referred to as the P6 loop) and so can be removed from the Group I intron without affecting its function leaving the Group I intron in two halves [12, 13]. The two halves of the intron are then permuted, that is, so that the second half now precedes the first half whilst retaining the 5′–3′ orientation of the sequences. As the structure and function of the intron is unchanged, *cis-splicing* occurs as before, only now the 3′ and 5′ exons circularize. Sequences of our choosing can then be inserted in between the 3′ and 5′ exon, permitting their circularization so long as they do not interfere with the group I intron architecture to a significant degree, and consequently enhancing their stability, whilst also hampering degradation by exonucleases [14, 15].

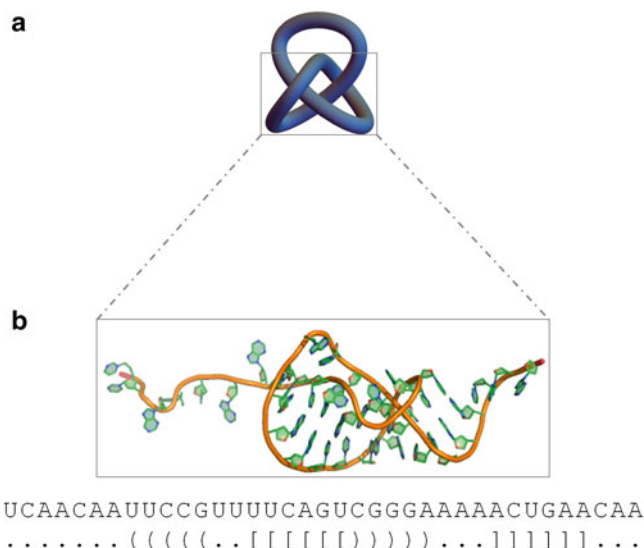


Fig. 1 Circularization of RNA “open” knots that produce topological knots. (a) An idealized schematic of a simple topological knot (in this instance, a trefoil knot). (b) A 3D visualization of an “open” knot of RNA produced in PyMol from estimates of 3D structure by RNAcomposer using the secondary structure delineated in *dot bracket* notation *below*. Circularization of such an open knot using the PIE method would result in a trefoil knot

The next tier of manipulation is the topological linking of two circular RNA, which has been previously described in literature as a two interlocking ring system. A study in 2008 [16] saw the creation of synthetic, functional RNA’s (termed RNA lassos) that could bond with a chosen target RNA molecule, and then circularize. The lassos are a novel class of antisense agents, consisting of two key features: a Hairpin Ribozyme, and an antisense target sequence. The antisense target region consists of short section of bases that are complementary to that of the target RNA molecule, encouraging the binding of the lasso to the target, and enabling the ribozyme to latch on to a chosen RNA molecule before circularizing around it. The lasso was originally designed for use as an antisense suppressor, targeting a selected nucleic acid sequence to inhibit interactions with other biomolecules, and providing a wide variety of uses within the gene expression market [12, 16]. Here however, it will be utilized as a tool to construct RNA architectures. For this design, the RNA lasso will target a self-assembled circular RNA, in this instance a circular product made using the PIE method, resulting in the formation of the two interlocking RNA rings. Prior experiments using an RNA lasso to target a circular RNA product synthetically formed using DNA ligase have proved successful, justifying the use of RNA lassos as building blocks for the higher tier systems [16].

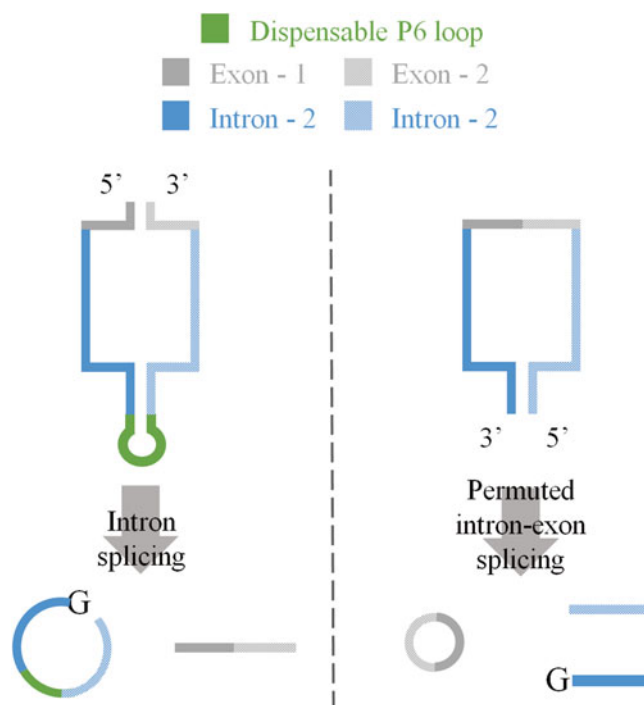


Fig. 2 Comparison of group I intron splicing and permuted intron–exon splicing. Normal intron splicing (*cis*-splicing) results in the ligation of the exonic regions, and can induce intron circularization. The permuted intron–exon (PIE) method rearranges the RNA sequences (in a fashion described as a cyclic permutation) so that the second half of the sequence precedes the first (with the dispensable P6 loop removed). Splicing of this sequence now produces a circularized exon, as desired. Note that these intron-splicing reactions occur spontaneously in the presence of a guanosine cofactor (such as GTP) and Mg^{2+} ions

The last tier of topological manipulation extends the two interlocking ring system using two RNA lassos to produce a string of topologically interlocked circles (an RNA chain) (depicted in Fig. 3a). Both lassos will have two antisense target regions, which will each be complementary to those on the remaining lasso. This will enable the lassos to interact in an alternating manner, assembling a chain of circular RNA. The RNA lasso used in the previous tier was redesigned for this system.

Previously the lasso comprised of three main components: the Hairpin ribozyme, a spacer, and the antisense target. This spacer provided the necessary flexibility for the lasso to bind to its target, circularize around it, and is engineered using a CAA nucleotide repeat [17]. The redesigned lasso, however, contains two spacers, and two different targets regions (which we refer to as TNF- α and PIE-target). TNF- α is a murine tumor necrosis factor that was

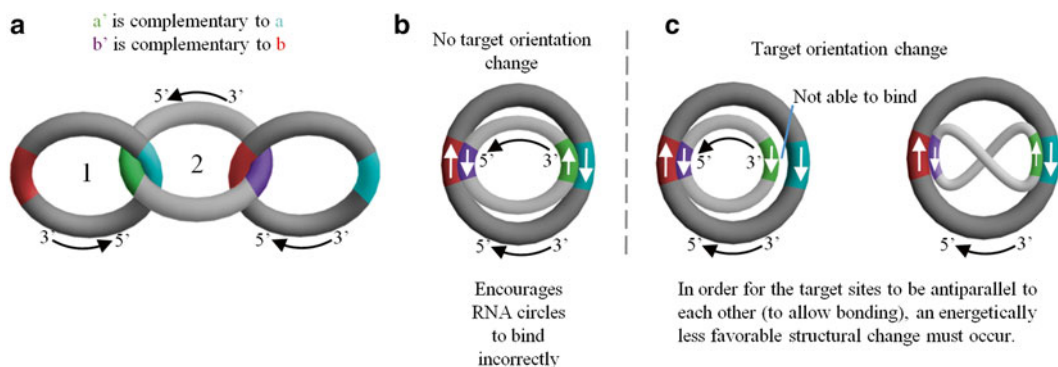


Fig. 3 Lasso interaction. (a) Demonstrates the way in which the two components of the chain system will interact, through an alternating target and lasso manor. (b) Depicts how double bonding of two lassos can occur when there is no target orientation change. Double bonding is when both targets of one lasso bind to both the targets of another. (c) Demonstrates how double bonding can be avoided through a target orientation change. Note *white arrows* depict target orientation to one another

originally targeted by an RNA lasso in the work by Dallas et al. [16] and the PIE-target is the same as target sequence used in the two interlocking ring system. A second spacer is included to avoid the possibility of one bound target sequence sterically interfering with the binding of the second target sequence [17]. If both target regions are not freely accessible the chain system will be less likely to form, and subsequently the two lassos are engineered so that only one target region will interact with one lasso. The lassos are designed with this constraint in mind, so as to prevent a situation where both targets on one lasso, bond to another lasso (Fig. 3b, c). To reduce the likelihood of this occurring, one of the target sequences runs in a different ($5'-3'$) orientation to the rest, in other words, its sequence is reversed. This change of orientation will disable double target binding, since one of the lassos would need to change to an energetically less favorable structure in order to form hydrogen bonds between the target sequences. It should be noted that this alteration would not impact the chain structure, as the molecules are free entities, and so can freely orientate themselves.

The RNA chain could theoretically form a necklace structure, should the terminating lasso bind to the first. Designing some lassos to contain more than two target sites, which we term “junction” lassos, would enable the creation of bifurcating structures. By altering the ratio of lassos and junction lassos, different end products could be obtained, opening up the possibility of a vast array of new nanostructures.

2 Materials

2.1 RNA Computational Design

Computational modelling should be used throughout the design process to assess the viability of the sequences. This modelling falls into two related categories: secondary structure and RNA–RNA interaction prediction. The latter is especially needed when considering the upper two tiers of topological manipulation, in pursuance of modelling the interaction of multiple RNA molecules.

2.1.1 Secondary Structure Prediction

Three webserver prediction tools were used to predict secondary structure (*see Note 1*):

1. NUPACK [18].
2. Vienna RNAfold program [19].
3. Kinefold [20].

2.1.2 RNA Interaction Prediction

The online server based RNA–RNA interaction models:

1. NUPACK [18].
2. Ribomaker [21].
3. Vienna's RNAcofold package [19].

Applying these in combination provides a means by which to validate results.

2.2 RNA Synthesis

Synthesize DNA of the designed sequence and perform in vitro transcription, using an appropriate kit. Tables 1 and 2 list the RNA sequences of parts used in our example constructs.

Restriction enzymes to linearize plasmid for in vitro transcription:
For interlocking lasso systems

1. PstI (FastDigest Thermo Scientific) with 10× FastDigest Buffer, knot
2. EcoRI (FastDigest Thermo Scientific) with 10× FastDigest Buffer, and for
3. PIE—Hind-III (BioLabs) using CutSmart Buffer

In vitro transcription:

TranscriptAid T7 High Yield kit (Thermo Scientific) (*see Note 2*), using the following buffer and reagents (Quantities given per 1 µg of DNA in 8 µL DEPC water): 2 µL TranscriptAid Buffer, 8 µL rNTPs mix (ATP, UTP, GTP, and CTP), 2 µL TranscriptAid Enzyme Mix.

2.3 RNA Characterization

2D-Denaturing Urea PAGE (Polyacrylamide Gel electrophoresis) involves running two gels:

2.3.1 2D-Denaturing Urea PAGE

1. 1D gel: 6 % polyacrylamide gel: 1× TBE, 8 M urea, 30 % acrylamide (19:1). Use 200 µL 10 % APS, and 10 µL TEMED to set gel.

Table 1
RNA sequences of lasso parts.

Name	Sequence	Ref.
Lasso	AGGUGUCCUCGUCCGUUAGACGAGAGAGGCCUACCAGAGAAACACACGACGUAAGUCGUGGUACAUU ACCUGGUACA *Spacer and target region(s)*CAAGGCUGUCCUCGUC <u>Underlined</u> regions are spliced out when the lasso circularizes.	[16]
TNF- α target	GUUCUCUUCAAGGGACAAGGCU	[16]
PIE-target	AUAAUUUGCCAUCUGGGU	[29]
Lasso Spacer	AACAACAACAAGAACACAACAAC	[16]
Lasso 1 spacers ^a	1. AACAAACAACUAAACAACAUACAACAAUCAAAC 2. AACAAACAACAUACAACAAUCAAACAAUCAAAC	[16]
Lasso 2 spacers ^a	1. AACUAACAACUAAACAACAACAACAACAAUCAAAC 2. AACAUACAACAACAACAAUCAAACAACAAUCAAAC	[16]
PIE (using group I intron found in T4)	GGUUCUACAUAAAUGCCUAAACGACUAUCCUUUUGGGAGUAGGGUCAAGUGACUCGAAACGAUAG ACAACUUUGCUUUAAACAAGUUGGAGAUAAUGUCUCUGCAUGGUGACAUGCAGCUGGUAUA AUCCGGGGUAAAGAUAAACGACCUUAUCUGAACAUAAUGCUACCGUUUAAUUAUUGGAUCC. *Aptamer* <u>CUGCAGAU GUUUUCUUUGGGUUAAUGAGGCCUGAGUAUAAAGGUGACUUAUACUUUG</u> <u>AAUCUAUCUAAAACGGGGAACCUUCUCUAGUAGACCAAUCCCGUGCUAAAUUGUAGGACUAAAGCUUC</u> UCGAG <u>Underlined</u> region denotes spliced out exon.	[29]
Streptavidin aptamer	CGACCAGAAUCAUGCAAGUGCGUAAGAUAGUCGGGGCCGG	[29]

Bold regions indicate regions where different parts can be inserted into the sequence

^aModified version of original lasso spacer

Table 2
RNA sequences of knot parts

Name	Sequence	Ref.
PIE (using group I intron found in <i>Graphium penicillioides</i>)	GGGGCCGUCCACAGACUAAGUGGUUGUGGGUAGGACGUCU GUUCUAUCUAAGAUUAAGUCGGGCCUUGCGAGAGAU CGC AGGGGUUUCUGCGUCCGUA *Aptamer* AAGUCGUAACAAG <u>GUUUUGCUAAGCAACAGUCCUUCUGUAGCAGAGCUUAAC</u> GGAGACCUUGCGCCAAAAGGCGACUAUAAAUAUUCGGCAG CAAGUCAGUAUCACUGGCGACACAAUCGAAUUGCGGGGAC GCUUUAAAAGCUCGACGGUACCAACCGCUGGAAAUCCAGC GGGGCUAGCAUGCUAGAGGUCAGAACUCGUCGAGAUGUA CAAUAAGCAAUCCGCGAGCGGUCCCC <u>Underlined</u> region denotes spliced out exon.	[29]
Knot aptamer ^a	UCAACAAUCCGUUUUCAGUCGGGAAAAACUGA ACAA (<i>see</i> Fig. 1)	[23, 24]

Bold regions indicate regions where different parts can be inserted into the sequence

^aFifth base change to a C (from a G) to reduce the likelihood of it binding with the intron

- 2D gel: 10 % acrylamide gel: 1× TBE, 8 M urea, 30 % acrylamide (19:1). Use 200 µL 10 % APS, and 10 µL TEMED to set gel.
- Urea gel loading buffer (Fisher Scientific).
- Ethidium bromide (EtBr).
- SCIE PLAS TV50 Single Gel Vertical Electrophoresis Unit.
- BioDocAnalyze Live (BioDoc Analyze Biometra) Gel Documentation Unit.

When characterizing the interlocking RNA systems the following buffers will be used:

- Magnesium Buffer—10 mM MgCl₂, 50 mM Tris-HCl (pH 7.5), and 20 % formamide. Make a 10× buffer containing MgCl₂ and Tris-HCl, then add formamide with the sample so that the concentration of formamide is 20 %.
- EDTA Buffer—10 mM EDTA, 50 mM Tris-HCl (pH 7.5), and 20 % formamide. Make in a similar fashion to magnesium buffer.

2.3.2 RNase R Treatment

RNase R (Epicentre) distributed with associated 10× RNase R Reaction Buffer. RNase R is a 3' → 5' exoribonuclease that digest linear RNAs but not circular or lariat RNA structures [25, 26] (*see Note 3*).

2.3.3 Reverse-Transcriptase-PCR (RT-PCR)

- RT: RNA sample, 2 µL (40 µM) Primer A (IDT) and 4 µL (10 mM) dNTPs (Thermo Scientific), 4 µL M-MuLV Reverse Transcriptase (New England Biolabs), 0.5 µL associated 10× M-MuLV buffer and 0.5 µL Ribolock (Thermo Scientific).

2. PCR: 2 μ L RT product, 2 μ L of Primers A and B (IDT), 0.4 μ L (10 mM) dNTPs (ThermoScientific), 0.6 μ L DMSO (BioLabs), and 4 μ L Phusion Polymerase (BioLabs).
3. Thermocycling done using TProfessional TRIO Thermocycler.
4. Gel Extraction: DNA Clean and Concentrator (Zymo Research) and ADB (Zymo Research).
5. Software ClusterW2 was used to perform sequence alignments.

3 Methods

3.1 RNA

Computational Design

3.1.1 Tier 1: *Topological Knot*

1. To find a suitable topological knot one can download over 1000 Group I sequences from the Group I Intron Sequence and Structure Database (GISSD) (categorized by subgroup here at <http://www.rna.whu.edu.cn/gissd/sequence.html>) [22]. Exon sequences are listed in lowercase characters and intron sequences in uppercase characters.
2. Amalgamate the each group I intron subgroup fasta file into one contiguous fasta file. Develop a script to convert this file into a format with the following fields:
 - (a) Intron name
 - (b) 5' exon sequence
 - (c) Intron sequence
 - (d) 3' exon sequence
3. To ensure that the “open” knot both forms and circularizes correctly, we aim to minimize interaction between the open knot sequence and the group I intron used to circularize it (via the PIE method). To select the most suitable intron for this task, use the following “indicators” (listed in order of importance):
 - (a) Flanking exons should not bind to the open knot sequence, as this could inhibit knot formation as well as interfere with the *cis*-splicing mechanism.
 - (b) The open knot sequence should not bind to the intron, as this too could interfere with knot formation, and prevent exon circularization.
4. To assess these criteria, use ViennaRNA package’s RNAcifold program [19]:
 - (a) Download and install/compile the ViennaRNA Package from: <http://www.tbi.univie.ac.at/RNA/index.html>
 - (b) The RNAcifold program is employed with the command: RNAcifold [OPTIONS]
Where instead of [OPTIONS] one inserts the two RNA sequences that interact, concatenated by the ampersand symbol (&), for example, CAUAA&GUGUA.

Multiple concatenated sequences can be imported into the program at once, and the results can be piped into a text file for further manipulation, for example, for Mac/Unix users:

RNAcofold CAUAA&GUGUA → exampleTextFile.txt

The software takes two RNA sequences and calculates the structure that has the lowest free energy (*see* **Note 4**).

5. Calculate the sum of the minimum free energies associated with: the 5' exon binding to the open knot, and the 3' exon binding to the open knot. This will serve as a numerical proxy for the first criterion. Similarly, adherence to the second criterion can be estimated by calculating the minimum free energy associated with the binding of the open knot sequence to the intron.
6. Select the group I intron with the best indicator value—the lowest free energies—then proceed to further evaluation.
7. Gauge whether both the group I intron and the open knot sequences fold correctly by checking the structure of these sequences, both independently and in combination, with the Vienna RNAfold package [19] and the ILM webserver (Kinefold) [18]. To avoid hybridization between the knot and the circularizing scaffold, model their interaction with RNAcofold. Should the knot and circularizing scaffold interact, restart the process with another circularizing scaffold from **step 5** (or consider choosing another knot sequence/making suitable point mutations).

3.1.2 Tier 2 and 3: Interlocking Structures

1. Model the secondary structure of the lasso with NUPACK:
 - (a) Access and adjust “RNA energy parameters” to “Matthews et al. 1999”.
 - (b) Adjust “Number of strand species” to 2 and “Maximum complex size” to amount required.
 - (c) Follow given instructions in help section of server.
2. Verify the following criteria:
 - The predicted secondary structure of the ribozyme should remain relatively intact, to increase the probability that it folds correctly.
 - The antisense target region should have least four bases accessible to act as a “Toehold”—regions that are single stranded in the target species [20].
 - Should a target region fold into a conformation where some of its base pairs are bound, the total predicted equilibrium probability of this binding should be small (at least below 0.5 base pair probability).

3. Validate results using Ribomaker:
 - (a) Access online server at: <https://absynth.issb.genopole.fr/Bioinformatics/tools/Ribomaker/>.
 - (b) Follow on screen instructions.

3.2 RNA Synthesis

3.2.1 In Vitro Transcription

1. Linearize plasmid using a suitable restriction enzyme for your construct (for our constructs, *see* Subheading 2 for a suitable enzyme).
2. Produce RNA in vitro using an appropriate transcription kit (e.g., TranscriptAid T7 High Yield kit from Thermo Scientific) (*see* **Note 5**).
3. Purify RNA by phenol–chloroform precipitation extraction [27]:
 - (a) Add to 20 μ L of transcription product 105 μ L DEPC water, and 15 μ L NaAC. Next add 140 μ L phenol–chloroform. Centrifuge for 5 min at 14,100 rcf.
 - (b) Extract top (aqueous) layer; add to this extraction 140 μ L chloroform. Centrifuge at 14,100 rcf for 5 min.
 - (c) Extract top layer, and add to this 280 μ L Ethanol (99.8 %). Store this overnight at -20°C .
4. Centrifuge for 30 min and discard resulting supernatant. Wash pellet with 500 μ L cold Ethanol (70 %).
5. Resuspend pellet in 25 μ L DEPC water.

3.3 Characterization of Circular RNA

3.3.1 2D Urea PAGE

2D Denature Urea PAGE (Polyacrylamide gel Electrophoresis) takes advantage of the change in migration speed seen in nonlinear topologies of nucleic acid when passing through differing polyacrylamide concentrations. Various topologies of nucleic acids can run on a one-dimensional gel without necessarily being distinguishable on the 1D gel alone (Fig. 6 gives example 1D gel result). Running the gel a second time, at a higher concentration of polyacrylamide, separates any circular RNA from the diagonal. This is because the resistance migrating through the polyacrylamide mix is higher for circular RNA than linear strands. Hence, linear RNA migrates approximately at the same speed as in the 1D gel, causing them to move into a diagonal line across the gel, leaving any circular RNA to protrude out from the diagonal (Fig. 4 depicts the trajectory taken by linear and topological RNAs on a 2D PAGE). An example of this procedure is outlined in Fig. 7.

1. Make 6 % polyacrylamide gel and run as normal (*see* **Notes 6 and 7**).
2. Once 1D gel is complete cut out column of interest using a scalpel.

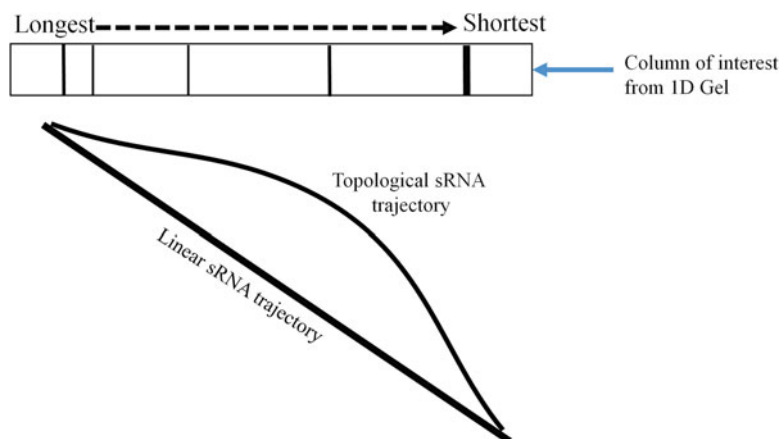


Fig. 4 Denaturing PAGE with RNAs from in vitro transcription of PIE sequence with streptavidin aptamer. Samples were separated by denaturing PAGE and the gel was stained with ethidium bromide. Lane 1, RNA marker with the sizes of the standards indicated on the *left* in nt; lane 2, RNA sample from in vitro transcription of PIE sequence with streptavidin aptamer, DNA plasmid expressing a self-circularized RNA [29]; and lane 3, RNA sample from in vitro transcription of PIE sequence with streptavidin aptamer treated with RNase R. The band containing circular RNA is indicated on the *right* with a *blue arrowhead*

3. Make second gel (10 % polyacrylamide) with room to place column horizontally above (*see* Fig. 4 and **Note 8**). Ensure there are no bubbles between the column and the gel before running.
4. Run the second gel; look for characteristic arc of nonlinear RNA (Figs. 4 and 7).

3.3.2 RNase R Treatment

Using this enzyme to treat the samples loaded into the gels above provides a clearer indication of the conformation of the RNA (Figs. 6 and 7).

1. Treat sample with RNase R, using a non-treated sample as a control. The amount of RNase R required is proportional to the amount of RNA you have; in 10 min at 37 °C one unit converts 1 µg of poly-r(A) into acid-soluble ribonucleotides.
2. To compare samples use a Urea PAGE—6 % polyacrylamide gel (using the same method outlined above) (Fig. 6).
3. The resulting columns from this gel can be carried forth into 2D gel (following the protocol outlined previously). In the second gel, the arc of the nonlinear RNA, and the differing brightness between the bands of linear and nonlinear RNA, indicate the presence of circular RNA (Fig. 7).

3.3.3 RT-PCR

Reverse-transcriptase PCR (RT-PCR) can be used to independently validate circular RNA detection via 2D PAGE. Comparison of two sequences can be achieved using ClusterW2 from <http://www.ebi.ac.uk/Tools/msa/clustalw2/> (Fig. 8). After the RT reaction, a PCR using divergent primers will only yield a band if the RNA tested is circular (Fig. 5 and *see* **Note 9**). Repetition of

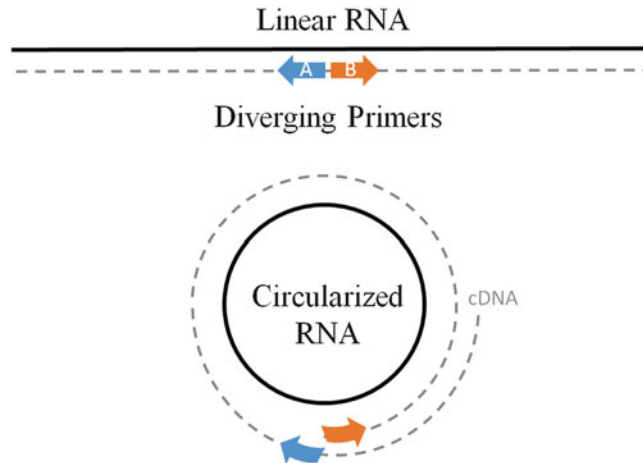


Fig. 5 Trajectories of circular and linear RNA in a 2D Denaturing Urea PAGE [7]

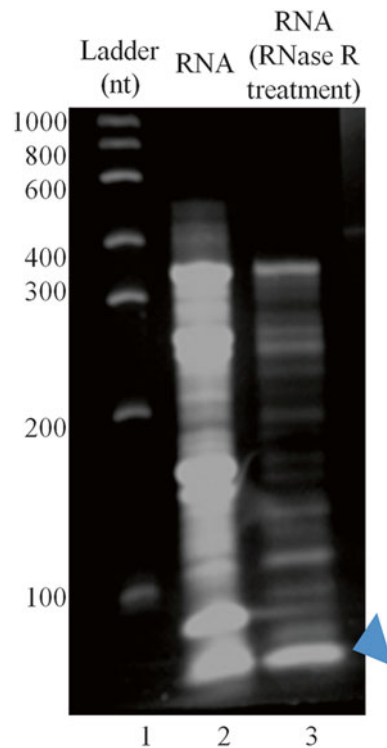


Fig. 6 2D Denaturing PAGE with RNAs from in vitro transcription of PIE sequence with streptavidin aptamer. Columns of 1D gel were separated by denaturing PAGE and the gel stained with ethidium bromide. (a) RNA sample from in vitro transcription of PIE sequence with streptavidin aptamer. (b) RNA sample from in vitro transcription of PIE sequence with streptavidin aptamer treated with RNase R. The positions of the circular RNA are indicated with a *blue arrowhead*

2D gels with RNAs from *in vitro* transcription with T7 RNA polymerase

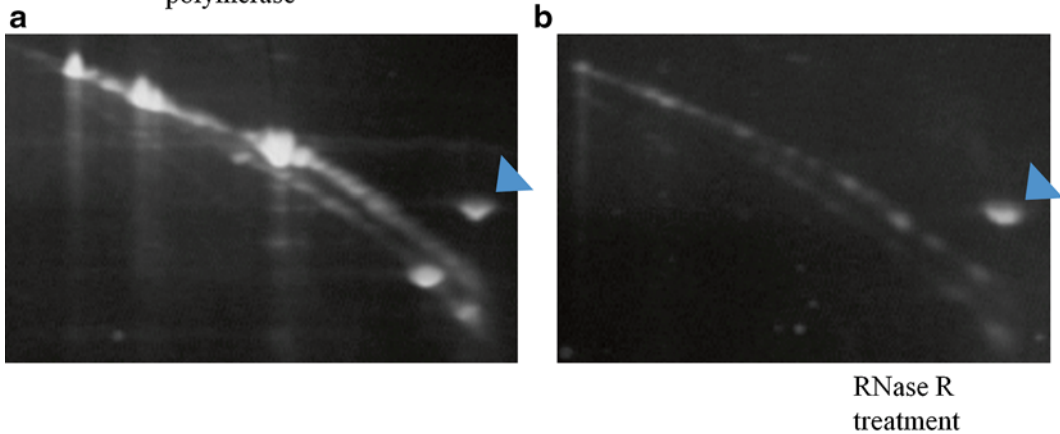


Fig. 7 RT-PCR DNA Sequencing of Circular RNA from PIE sequence with streptavidin aptamer. The figure shows the alignment between the theoretical sequence of the circular RNA, formed by the PIE-circularization of the T4 bacteriophage and Streptavidin aptamer sequence, and the sequence obtained by RT-PCR. The primer for the RT is shown in *yellow*, and periodic repetition of the circular RNA sequence occurs as expected

```

Predicted Repeat Region CGCACTTGCATGATTCTGGTCGGGGATCCAATATTAAACGGTAGACCCAAGAAAACATCT
RT-PCR                  -----GC-TAATTT-----AACGGTAGACC--AAGAAACATCT
                        ** * ** *                               * * * * *

Predicted Repeat Region GCAGCCGGCCCGCGACTATCTTAACGCACTTGCATGATTCTGGTCGGGGATCCAATATTAA
RT-PCR                  GCAGCCGGCCCGCGACTATCTTAACGCACTTGCATGATTCTGGTCGGGGATCCAATATTAA
                        *****

Predicted Repeat Region ACGGTAGACCCAAGAAAACATCTGCAGCCGGCCCGCGACTATCTTAACGCACTTGCATGAT
RT-PCR                  ACGGTAGACCCAAGAAAACATCTGCAGCCGGCCCGCGACTATCTTAACGCACTTGCATGAT
                        *****

Predicted Repeat Region TCTGGTCGGGGATCCAATATTAAACGGTAGACCCAAGAAAACATCTGCAGCCGGCCCGCG
RT-PCR                  TCTGGTCGGGGATCCAATATTAAACGGTAGACCCAAGAAAACATCTGCAGCCGGCCCGCG
                        *****

Predicted Repeat Region ACTATCTTA-
RT-PCR                  ACTATCTTACG
                        *****

```

Fig. 8 Schematic of RT-PCR with divergent primers on linear and circular RNAs. *Blue* and *orange* arrows indicate direction of the two primers on cDNA (shown in *grey*) [7]

the circular RNA sequence will be present in the cDNA, indicating that the RNA is circular. A gel is able to detect the number of repetitive periodic sequences generated by the RT-PCR can be detected on a 1D gel via separation by molecular weight [1].

1. Design a primer for the RT and back-to-back diverging primers for the PCR (Fig. 5).
2. Prepare RT, add sample to primer A and dNTPs. Heat this solution to 65 °C for 5 min then centrifuge for 3–5 s at 14,100 rcf. Place directly on ice once centrifuged.

3. Add to solution Ribolock, M-MuLV Reverse Transcriptase and its associated buffer. Using a Thermocycler, heat the solution to 50 °C for 1 min (*see Note 10*), after which raise to 85 °C for 5 min, and finally 65 °C for 20 min [29].
4. Run a standard PCR with reagents outlined in Subheading 2.
5. Product can be visualized on a 1 % agarose gel; each band will correspond to the number of DNA repeats of the circular RNA.
6. The bands can be extracted from the gel and sequenced with either of the primers to ensure circularization and correct ligation site. Extraction Protocol:
 - (a) Weigh gel and add 100 mg of gel for every 300 μ L ADB.
 - (b) Heat to 55 °C for 10 min (shake after 5 min).
 - (c) Transfer solution to a spin column and spin down for 1 min. Discard supernatant.
 - (d) Add 200 μ L Washing buffer to the column and spin down for 1 min. Discard supernatant and repeat this step.
 - (e) Spin for 1 min to remove residual wash. Transfer column to a new 1.5 mL tube.
 - (f) Add 9 μ L Elution buffer, then incubate for 2 min.
 - (g) Spin solution down and dispose of column.

3.4 Characterization of Two Interlocking Rings and Chain System

3.4.1 1D Urea PAGE

RNA lassos needs Mg^{2+} ions to pass between linear and circular conformations, and can be tested in both buffers (one with magnesium and one with EDTA) to characterize the interlocking RNA complexes. The EDTA acts as a sink removing any magnesium present in the solution.

There are four conditions that the lasso and target should operate under to be confident in the characterization of the interlocking system. Run each of the four conditions in separate wells, for both buffers, on a 1D PAGE (Subheading 3.3.1).

1. *Without the target present:* 7 μ L lasso, 2 μ L buffer, 4 μ L formamide (100 %), and 7 μ L H_2O . This demonstrates the location of the different lasso forms (linear, half processed and circular) when run on 1D PAGE, so that they can be ruled out in later lanes. For the chain system, run the two lassos in separate lanes in the specified solution.
2. *Lasso with the target (or other lasso) present:* 7 μ L lasso, 7 μ L target (or other lasso), 2 μ L buffer, and 4 μ L formamide (100 %). After the 1D gel has run, some bands will be seen at different molecular weights for the two buffer solutions. At this stage it will not be clear which bands contain the interlocking rings, and which are simply molecular dimers (or multimers).
3. *Lasso with the target (or other lasso) present following heat treatment:* 7 μ L lasso, 7 μ L target (or other lasso), 2 μ L buffer, and

4 μL formamide (100 %). Before the Urea loading buffer is added, heat the solutions to 95 °C. Any of the complexes formed between the molecules that are not interlocked will be lost, supporting the case for one band containing interlocking complexes over another.

4. *Lasso with the target (or other lasso) present in RNase R*: 7 μL lasso, 7 μL target (or other lasso), 2 μL buffer, and 4 μL formamide (100 %). Before Urea loading buffer is added, treat with RNase R (*see* Subheading 3.3.2). After this treatment only the interlocking complexes and circular RNA should be present, enabling the distinction of the interlocking system.

3.4.2 Reverse Transcription-PCR (RT-PCR)

Once detected in the gel-based tests, the band believed to be the two-ring interlocking system can be validated using RT-PCR. Two RT-PCRs can be conducted on the sample to verify the presence of both components (of the two interlocking rings or chain system). If both RT-PCRs indicate the presence of the component, it follows that the eluted product is an interlocking complex.

1. Elute out the band presumed to containing the two interlocking rings or chain system.
2. As in the previous RT-PCR design, divergent primers are essential (*see* Subheading 3.3.3 and Fig. 5). Primers should be specific to each lasso/circular target.
3. Run RT-PCR as before, with each set of primers run separately.
4. If both RT-PCRs indicate the presence of the component, it follows that the eluted product is an interlocking complex.

4 Notes

1. NUPACK and RNAfold are based on deterministic models and generate results for shorter sequences almost instantaneously. However, they exclude the possibility of pseudoknots when generating minimum free energy structures for sequences smaller than 100 bp, and Xayaphoummine, Bucher and Isambert [20] found that there is practically no correlation between predictions for low energy structures including and excluding pseudoknots. Kinefold predictions are stochastically generated, and the webserver can handle 100+ bp sequences that may fold into pseudoknots, but it is currently more difficult to extract secondary structure information from. It is advised to run simulations multiple times using different random seeds in order to validate results.
2. If a T7 or SP6 promoter is used, remember to add a G at the 5' of the RNA during secondary structure, as it is part of the

promoter but transcribed by the polymerase. T7 and SP6 may also add a base at the 3' of the RNA.

3. Ribonuclease R (RNase R) is an exoribonuclease that digests linear RNAs. It does not however digest lariat or circular RNA structures, or double stranded RNA with 3' overhangs shorter than seven nucleotides. Most cellular RNAs will be digested completely by RNase R, with the exception of tRNAs, 5S RNA, and intron lariats [30]
4. At the time of writing, RNAcofold appears to estimate the free binding energy between two separate RNA strands by concatenating the strand into one longer strand before finding the minimum free energy of this new structure using the RNAfold algorithm. If this is the case, RNAcofold is inappropriate for investigating interactions between small sequences, but still serves as a good indicator of binding energies for longer sequences [28], especially in consideration of the ease with which data can be imported and exported using RNAcofold. Subsequently, it is advised that steps are taken to validate these binding energies using alternative algorithms following the initial filtering process.
5. PIE products are incubated for 30 min at 55 °C after the transcription to allow the products to circularize. This heating encourages self-ligation of the flanking exons during splicing [29].
6. The PAGE gels can sometimes run out from the bottom of plates when setting. To avoid this, one can set the bottom of the gel by taking 500 µL gel mix solution and adding to this 1 µL TEMED, and 8 µL APS. This will quickly begin to polymerize, so immediately add the solution between the gel plates. This solution will set much faster than the main gel, and so will seal the bottom of the plates before the PAGE gel slips out.
7. To improve the quality of PAGE gels, a pre-run (running the gel with no sample) is recommended as it reduces the “smiling effect” sometimes seen in these gels.
8. Placing the gel column horizontally on top of the new gel can be difficult. If the column gets stuck, or shows signs of resistance when being moved, add water to lubricate gel.
9. Owing to the elaborate secondary structure of the molecules tested with RT-PCR, the yield of the RT-PCR reaction can be low. This is because the secondary structure of these molecules can be complicated, with many loops, resulting in the RNA polymerase sometimes falling off the RNA, reducing the overall yield.
10. Longer incubation times during reverse transcription using RNase H deficient reverse transcriptases will result in a higher number of repeats of the sequence within the resulting DNA. This is due to the transcriptase looping around the RNA circle, but producing linear DNA.

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Folding RNA–Protein Complex into Designed Nanostructures

Tomonori Shibata, Yuki Suzuki, Hiroshi Sugiyama, Masayuki Endo, and Hirohide Saito

Abstract

RNA–protein (RNP) complexes are promising biomaterials for the fields of nanotechnology and synthetic biology. Protein-responsive RNA sequences (RNP motifs) can be integrated into various RNAs, such as messenger RNA, short-hairpin RNA, and synthetic RNA nano-objects for a variety of purposes. Direct observation of RNP interaction in solution at high resolution is important in the design and construction of RNP-mediated nanostructures. Here we describe a method to construct and visualize RNP nanostructures that precisely arrange a target protein on the RNA scaffold with nanometer scale. High-speed AFM (HS-AFM) images of RNP nanostructures show that the folding of RNP complexes of defined sizes can be directly visualized at single RNP resolution in solution.

Key words Ribonucleoprotein, Kink-turn RNA, L7Ae, RNA–protein complex, RNA nanostructures, RNA nanotechnology, RNP, High-speed atomic force microscopy

1 Introduction

Naturally occurring RNP complexes play an important role in modulating the structure and function of RNA molecules, which regulate a variety of cellular events [1–3]. For example, the bacterial ribosome consists of three ribosomal RNAs and approximately 50 ribosomal proteins that form complex RNP structures through a set of RNP interactions [4, 5]. Their sophisticated structure and function render RNP motifs as promising building blocks for a wide range of applications including synthetic biology and nanotechnology. Integration of RNP motifs into functional RNAs allows us to construct artificial RNAs with protein-controllable folding and function. We have previously reported that RNP complex-mediated nanostructures can be designed and constructed using RNA kink-turn (K-turn) motifs and ribosomal L7Ae protein [6]. The K-turn–L7Ae interaction bends the RNA motif by approximately 60° [7–10], leading to the formation of

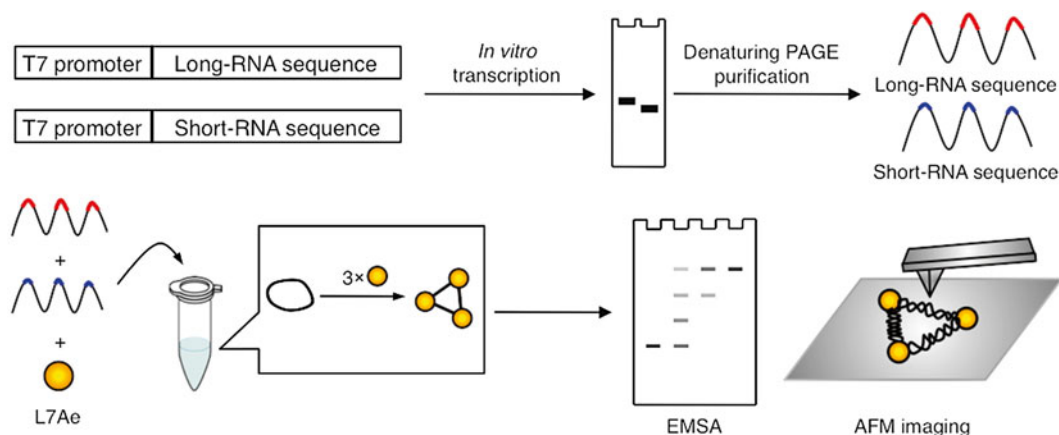


Fig. 1 Schematic illustration of the preparation and evaluation of RNP nanostructures. The RNP interaction can be evaluated by EMSA. The folding of RNP nanostructures in solution can be directly visualized by HS-AFM

equilateral triangular RNP nanostructures. In principle, desired functional proteins conjugated with L7Ae can be precisely arranged on a designed RNA scaffold of optimal size. Such RNP nanostructures, when attached to a functional protein module, can be used to detect mammalian cells [11]. Direct observation of native RNP interactions at high resolution is important for understanding the RNP folding and development of functional RNP nanostructures. HS-AFM is a powerful tool for capturing the molecular events of biological macromolecules such as DNA–protein interactions at high resolution [12–15]. Thus, we used HS-AFM to observe the folding of the RNP complex into nanostructures at single RNP resolution and precise arrangement of proteins on RNA nanostructures of defined sizes.

In this chapter, we describe a strategy to construct and visualize equilateral triangular RNP nanostructures of different sizes. The size of the RNP nanostructures can be easily tuned by changing the length of the double helix between three K-turn motifs that are incorporated into the three vertices of the triangle. These RNP nanostructures can be analyzed biochemically and visualized directly by electrophoretic mobility shift assay (EMSA) and HS-AFM, respectively (Fig. 1).

2 Materials

2.1 RNA synthesis

1. DNA templates and primers.
2. DNA polymerase: KOD-Plus-ver.2 (Toyobo, Japan).
3. Thermal cycler.
4. DNA purification kit: QIAquick PCR purification kit (Qiagen, Germany).

5. UV spectrometer: NanoDrop (Thermo Scientific, USA).
6. RNA polymerase: MEGAscript T7 kit (Amibion, USA).
7. Electrophoresis apparatus.
8. Electrophoresis buffer: 0.5× TBE (Tris-Borate-EDTA buffer).
9. Denaturing polyacrylamide gel: 10 % acrylamide-bis mixed solution (19:1), 8.3 M urea, 0.5× TBE.
10. Gel Indicator RNA Staining Solution, 100× (BioDynamics Laboratory Inc., Japan).
11. Elution buffer: 0.3 M NaOAc (pH 5.2), 0.1 % SDS.
12. Phenol, saturated with TE buffer (Nacalai Tesque, Japan).
13. Diethyl ether.
14. Ethanol.
15. 80 % Ethanol.
16. 3 M sodium acetate.
17. Milli-Q water (Millipore, USA).

2.2 Protein Synthesis

1. pET-28b(+)-L7Ac plasmid.
2. Competent cell: Rosetta(DE3) pLysS pRARE2.
3. Luria-Bertani (LB) medium.
4. Kanamycin.
5. Chloramphenicol.
6. 1 M isopropyl β -D-thiogalactoside (IPTG).
7. Shaking incubator.
8. Sonicator.
9. Centrifuge.
10. Ni-NTA Superflow resin (Qiagen).
11. Lysis buffer: 20 mM Tris-HCl buffer (pH 7.6), 300 mM NaCl.
12. Wash buffer: 20 mM Tris-HCl buffer (pH 7.6), 300 mM NaCl, 10 mM imidazole.
13. Elution buffer: 20 mM Tris-HCl buffer (pH 7.6), 20 mM NaCl, 500 mM imidazole.
14. SDS-PAGE apparatus.
15. SDS running buffer: 25 mM Tris-Base, 191 mM glycine, 0.1 % SDS.
16. Dialysis system.
17. Dialysis buffer: 20 mM HEPES-KOH (pH 7.8), 150 mM KCl, 1.5 mM MgCl₂, 5 % glycerol.
18. Stock buffer: 20 mM HEPES-KOH (pH 7.8), 150 mM KCl, 1.5 mM MgCl₂, 40 % glycerol.

19. Bradford reagent: Bio-Rad Protein Assay (Bio-Rad, USA).
20. Microplate reader (Tecan, Switzerland).

2.3 EMSA

1. RNA solution (adjusted to 1 μ M with Milli-Q water).
2. Protein stock buffer: 20 mM HEPES–KOH (pH 7.5), 150 mM KCl, 1.5 mM MgCl_2 , and 40 % glycerol.
3. L7Ae protein solution (adjusted to 10 $\times$ concentration with protein stock buffer).
4. 5 $\times$ Binding buffer: 100 mM HEPES–KOH (pH 7.5), 750 mM KCl, 7.5 mM MgCl_2 .
5. Thermal cycler.
6. Gel loading buffer: 0.25 % bromophenol blue (BPB), 30 % glycerol.
7. Electrophoresis apparatus.
8. Electrophoresis buffer: 0.5 $\times$ TBE.
9. Native polyacrylamide gel: X% Acrylamide–Bis Mixed Solution (19:1), 0.5 $\times$ TBE.
10. SYBR Green I and II (Lonza, Switzerland).
11. Gel image scanner: Typhoon FLA-7000.

2.4 High-Speed Atomic Force Microscopy (HS-AFM)

1. Mica disks with a diameter of 1.5 mm (Furuuchi Chemical Corporation, Tokyo, Japan).
2. 3-Aminopropyltriethoxy silane (APTES) for mica functionalization.
3. Ultrapure water for mica functionalization.
4. Solution of 50 nM RNP nanostructures prepared for the EMSA procedure (Subheading 3.3).
5. Imaging buffer: 20 mM Tris–HCl (pH 7.6), 10 mM MgCl_2 .
6. High-speed atomic force microscopy (Nano Live Vision, RIBM, Japan).
7. Cantilever (BL-AC10EGS, Olympus, Japan).

3 Methods

3.1 RNA Synthesis

All RNAs are synthesized by in vitro transcription using template DNAs and purified by denaturing PAGE (Fig. 1). The molecular design of a RNP nanostructure with an equilateral triangular shape has been reported [6, 11]. We designed equilateral-triangular RNP nanostructures of five different sizes (Fig. 2). The triangles have lengths of 15, 26, 48, 70, or 92 base pairs of double stranded RNAs that consist of long-RNA (L-RNA) and short-RNA (S-RNA). Hybridization between L-RNA and S-RNA leads to the

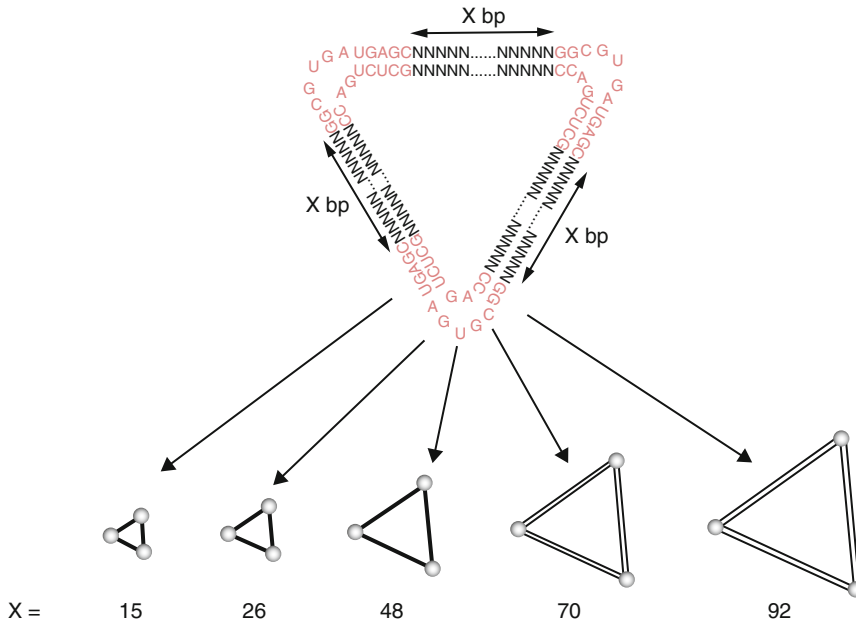


Fig. 2 Design of RNP nanostructures of different sizes. The equilateral-triangular RNP nanostructures consist of three K-turn motifs and three RNA duplexes. The number of base pairs in the RNA duplexes can be easily changed to construct RNP nanostructures of different sizes. The length of the three sides, which contain the stem region of the K-turn motifs, is designed to be a multiple of 11

formation of three K-turn motifs-containing RNA nanostructures (LS-15, LS-26, LS-48, LS-70, and LS-92).

1. Synthesize template DNA using DNA polymerase (Toyobo, Japan) by PCR (*see Note 1*).
2. Electrophorese the reaction mixture with native polyacrylamide gel to confirm template DNA amplification.
3. Purify the PCR product using QIAquick PCR purification kit.
4. Measure the DNA concentration using NanoDrop.
5. Transcribe DNA template using MEGAshortscript T7 kit. In a final volume of 20 μ L, mix Nuclease-free water, 150 nM DNA template, 2 μ L of 10 $\times$ Reaction buffer, 2 μ L of 75 mM ATP solution, 2 μ L of 75 mM UTP solution, 2 μ L of 75 mM GTP solution, 2 μ L of 75 mM CTP solution, and finally add 2 μ L of T7 Enzyme mix. The reagents used here are accessories in MEGAshortscript T7 kit.
6. After incubation at 37 $^{\circ}$ C for 4 h, add 1 μ L of TURBO DNase (contained in MEGAshortscript T7 kit) and incubate at 37 $^{\circ}$ C for 30 min.
7. Add 21 μ L of Gel Loading Buffer II (contained in the MEGAshortscript T7 kit) and incubate at 95 $^{\circ}$ C for 3 min.

8. Electrophorese the sample with denaturing PAGE containing 8.3 M urea and 0.5× TBE.
9. Stain the gel with 1× Gel Indicator RNA Staining Solution until RNA bands appear (*see* **Note 2**).
10. Cut the band of the desired RNA and then crush the excised gel.
11. Add 600 μ L of Elution buffer to the gel and then incubate at 37 °C for more than 2 h (*see* **Note 3**).
12. Recover the supernatant. Wash the recovered supernatant with 600 μ L of TE-saturated phenol at three times and with 900 μ L of diethyl ether.
13. Add 1,200 μ L of 100 % ethanol and 50 μ L of 3 M sodium acetate and then precipitate the RNA at –30 °C for more than 1 h.
14. Centrifuge at 20,400×*g* for 30 min, remove the supernatant, and wash the pellet with 600 μ L of 80 % ethanol.
15. After drying in air, dissolve in Milli-Q water. Measure the RNA concentration using NanoDrop.
16. Check the size and purity of the recovered RNA by denaturing PAGE and confirm the hybridization between L-RNA and S-RNA by native PAGE.
17. Store the RNA at –30 °C.

3.2 Protein Synthesis

L7Ae was prepared by conventional methods for protein preparation. The expression of L7Ae protein was performed by the transformation of *E. coli*. The L7Ae protein can be purified by affinity chromatography using polyhistidine-tag (His-tag).

1. Transform Rosetta(DE3) pLysS pRARE2 with pET-28b(+)-L7Ae plasmid.
2. Pick up a colony and then culture transformed *E. Coli* in 50 mL of LB medium containing 50 μ L of 50 mg/mL kanamycin and 15 μ L of 15 mg/mL chloramphenicol at 37 °C for >12 h.
3. Add 25 mL of the culture to 250 mL of LB medium containing 50 μ g/mL kanamycin and incubate at 37 °C until OD₆₆₀ reaches 0.5.
4. Add 250 μ L of 1 M IPTG to the culture and incubate at 37 °C for 4 h.
5. Centrifuge the cells at 4,100×*g* for 10 min and remove the supernatant.
6. Add 5 mL of Lysis buffer and then suspend the collected cells.
7. Sonicate the suspended cells on ice.
8. Heat the lysate at 80 °C for 15 min to denature endogenous proteins (*see* **Note 4**).

9. Centrifuge at $15,000\times g$ for 30 min and then filter the supernatant with a $0.45\ \mu\text{m}$ filter.
10. Wash Ni-NTA resin with Lysis buffer and then load the supernatant onto the Ni-NTA resin-filled column.
11. Wash the resin with Wash buffer to elute proteins without His-tag.
12. Add $100\ \mu\text{L}$ of $0.2\ \text{N}$ NaOH to the column and then immediately wash the resin with Wash buffer (*see* **Note 5**).
13. Elute with Elution buffer.
14. Collect fractions and check the fraction containing L7Ae proteins by SDS-PAGE.
15. Dialyze the purified L7Ae protein solution in Dialysis buffer at three times.
16. Measure the concentration of L7Ae by the Bradford method.
17. Check the binding activity of L7Ae to box C/D K-turn RNA by EMSA.
18. Store the L7Ae protein in Stock buffer at $-30\ ^\circ\text{C}$ for short-term storage or at $-80\ ^\circ\text{C}$ for longer storage.

3.3 Electrophoretic Mobility Shift Assay (EMSA)

EMSA is a general tool for the investigation of the interaction between biological macromolecules. The formation of RNA-protein nanostructures can be analyzed by EMSA. Hybridization between L-RNA and S-RNA should form three box C/D K-turn motifs capable of binding to L7Ae. LS-RNAs of various sizes were assayed by EMSA in the absence and presence of L7Ae (Fig. 3).

1. Prepare native polyacrylamide gel (*see* **Note 6**) and set up the electrophoresis apparatus. Perform a pre-run at constant voltage.
2. Mix $6\ \mu\text{L}$ of Milli-Q water, $2\ \mu\text{L}$ of $5\times$ Binding buffer, $0.5\ \mu\text{L}$ of each RNA solution (final concentration $50\ \text{nM}$).
3. After heating at $80\ ^\circ\text{C}$ for 3 min, leave at room temperature for 10 min.
4. Add $1\ \mu\text{L}$ of $10\times$ L7Ae protein solution and incubate at room temperature for 30 min.
5. Add $2\ \mu\text{L}$ of $6\times$ loading dye and load the sample in the polyacrylamide gel. Electrophorese at constant voltage for an appropriate time.
6. Detach the gel from the electrophoresis apparatus and stain the gel with SYBR Green I and II (Lonza, Switzerland) for 10 min (*see* **Note 7**).
7. Observe the gel with Typhoon FLA-7000 (*see* **Note 8**).

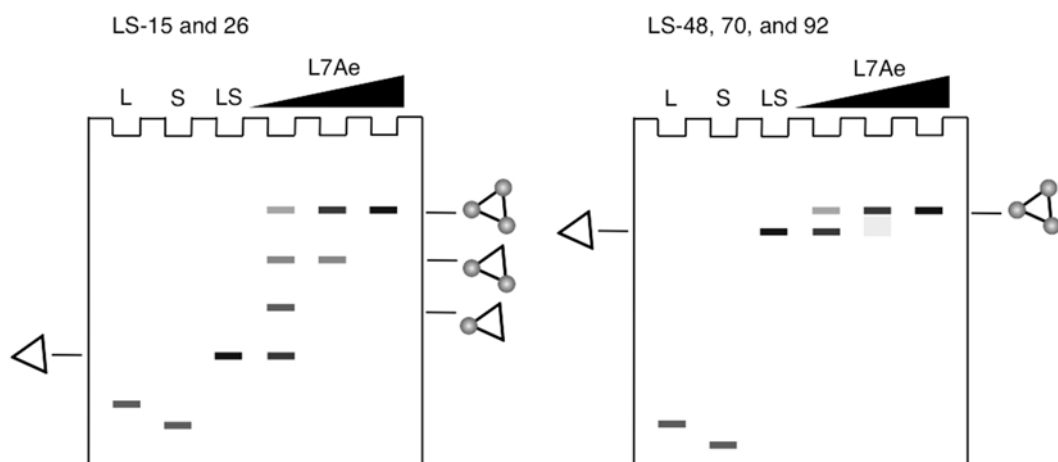


Fig. 3 EMSA of RNP nanostructures. L- and S-RNAs interact with each other to form LS-RNAs of circular structures. In the case of LS-15 and 26, three up-shifted bands are observed in an L7Ae concentration-dependent manner (*left*). The multiple RNP interactions of larger nanostructures (LS-48, 70, and 92) are indistinguishable by EMSA (*right*)

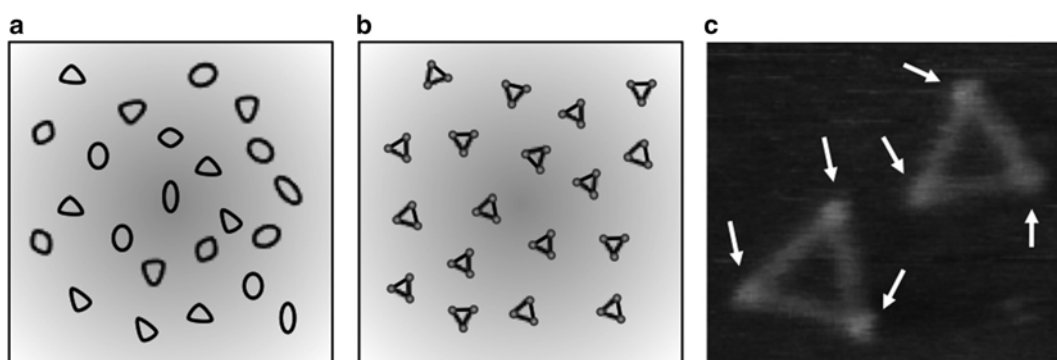


Fig. 4 Schematic illustration of AFM image (a) before and (b) after the addition of L7Ae. RNA nanostructures are heterogeneous structures in the absence of L7Ae due to flexibility of the K-turn motifs. However, the three K-turn motifs interact with L7Ae and bend by approximately 60° , leading to the formation of triangular RNP nanostructures. (c) Direct observation of RNP nanostructures in solution using HS-AFM. Single RNP interaction in the nanostructures is clearly observed (*white arrows*)

3.4 High-Speed Atomic Force Microscopy (HS-AFM)

3.4.1 Preparation of Mica Surface

The obtained RNP nanostructures can be visualized by AFM, which is now a standard technique for visualizing nucleic acids nanostructures. We employed HS-AFM (Nano Live Vision, RIBM, Japan), which enables quick data acquisition of high-resolution images in liquid (Fig. 4). The following sections describe the preparation of samples for AFM imaging.

1. Prepare a 0.1 % APTES solution in ultrapure water.
2. Cleave the mica disc to obtain a fresh surface.

3. Deposit a drop ($\sim 2 \mu\text{L}$) of 0.1 % APTES solution onto the freshly cleaved mica surface (*see Note 9*).
4. Rinse the surface with ultrapure water ($\sim 10 \mu\text{L}$) after incubation for 5 min at room temperature (25°C).

3.4.2 Sample Preparation for AFM Imaging

1. Dilute the pre-formed RNP nanostructures to $\sim 5 \text{ nM}$ by adding Imaging buffer (*see Note 10*).
2. Place $\sim 2 \mu\text{L}$ of the sample solution onto the APTES-treated mica surface for 5 min.
3. Rinse the surface with Imaging buffer ($\sim 10 \mu\text{L}$) to remove unbound molecules.

3.4.3 AFM Imaging of the Samples

1. Place the cantilever on the cantilever holder (*see Note 11*).
2. Fill the liquid cell with $\sim 120 \mu\text{L}$ of Imaging buffer.
3. Align the laser focusing position so that the intensity of the laser light reflected back from the cantilever is maximized.
4. Align the photodetector position so that the reflected laser makes a spot at the center of the photodetector.
5. Mount the sample prepared in Subheading 3.4.2 on the scanner stage.
6. Mount the scanner over the liquid cell in which the cantilever is immersed in Imaging buffer.
7. Find the resonant frequency of the cantilever using a fast Fourier transform (FFT) analyzer.
8. Excite the cantilever at the resonant frequency by applying sinusoidal AC voltage.
9. Execute the approach until the software automatically stops the motor.
10. Adjust the set point voltage to $\sim 75\text{--}95\%$ of the free oscillation amplitude.
11. Gradually decrease the set point voltage until the sample is clearly imaged (*see Note 12*).

4 Notes

1. We prepared double-stranded DNA templates for transcription by PCR. DNA templates of L-15, L-26, L-48, S-15, S-26, and S-48 are synthesized from two single-stranded DNAs with the corresponding sequences. In the cases of L-70, L-92, S-70, and S-92, these DNA templates are synthesized from four single-stranded DNAs through two-step PCR. The primer sequences have been reported [11].

2. Gel Indicator RNA Staining Solution can detect transcribed RNAs without UV irradiation. The staining time is 10–30 min, but depends upon the quantity of RNA.
3. Elution time depends on the length of RNA. When longer RNAs are eluted, we recommend incubation at 37 °C for more than 12 h (overnight) to maximize the recovery of RNAs.
4. Since L7Ae has thermostable, only endogenous proteins in *E. coli* are denatured and aggregated by heating.
5. The treatment of NaOH allows for the removal of contaminated nucleic acids in L7Ae. Contamination affects specific RNA–protein interactions and nucleic acid staining in EMSA. Nucleases can also be used to remove the contaminated nucleic acids.
6. The concentrations of the native polyacrylamide gels are changed in response to the size of the RNA and RNP nanostructures (LS-15, 10 %; LS-26, 7.5 %; LS-48, -70, and -92, 5 %).
7. The staining efficiency of RNA with SYBR Green I and II will be influenced by the RNA size, structures, and RNP interaction. Expanding the size of the RNA nanostructures will increase the staining efficiency.
8. Three up-shifted bands can be observed in small RNA nanostructures (Fig. 3 left; LS-15 and LS-26) depending on the L7Ae concentration. One up-shifted band is clearly observed in large RNA nanostructures (Fig. 3 right; LS-48, LS-70, and LS-92). This may be due to insufficient separation under our electrophoretic conditions.
9. APTES is used to coat the negatively charged mica surface. The treatment of APTES provides a positively charged mica surface, which immobilizes RNP nanostructures on the mica surface by electrostatic interactions.
10. The pre-formed RNP nanostructures are prepared using a procedure similar to that described in EMSA.
11. For HS-AFM imaging, small cantilevers are used. Small cantilevers (9 μm long, 2 μm wide and 130 nm thick; BL-AC10DS, Olympus, Tokyo, Japan) made of silicon nitride with a spring constant of ~0.1 N/m, and a resonant frequency of ~300–600 kHz in water and ~1,500 kHz in air are commercially available from Olympus. These cantilevers have bird beak-like tips, however, the apex of the tips are not sharp enough to obtain high-resolution images of RNP nanostructures in solution. Therefore, we use custom-made cantilevers having electron-beam deposited (EBD) tips at the top of the bird beak-like tips (BL-AC10EGS, Olympus, Tokyo, Japan).
12. The samples are imaged in Imaging buffer with a scanning rate of 0.2–1.0 frame/s (fps).

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Chapter 15

Simple Method for Constructing RNA Triangle, Square, Pentagon by Tuning Interior RNA 3WJ Angle from 60° to 90° or 108°

Emil F. Khisamutdinov, My Nguyen Hoan Bui, Daniel Jasinski, Zhengyi Zhao, Zheng Cui, and Peixuan Guo

Abstract

Precise shape control of architectures at the nanometer scale is an intriguing but extremely challenging facet. RNA has recently emerged as a unique material and thermostable building block for use in nanoparticle construction. Here, we describe a simple method from design to synthesis of RNA triangle, square, and pentagon by stretching RNA 3WJ native angle from 60° to 90° and 108°, using the three-way junction (3WJ) of the pRNA from bacteriophage phi29 dsDNA packaging motor. These methods for the construction of elegant polygons can be applied to other RNA building blocks including the utilization and application of RNA 4-way, 5-way, and other multi-way junctions.

Key words RNA nanotechnology, RNA nanoparticles, *In silico* design, pRNA 3WJ

1 Introduction

RNA nanotechnology has seen rapid growth as a new platform since 1998 when the concept was first introduced [1]. The ability to manipulate RNA molecules to fabricate nanometer-scale particles with predictable geometrical and functional properties has resulted in a renewed interest in nanosized material construction and nanotechnology including optical sensing [2–4], delivery of therapeutics [5–12], computer design [13, 14], and intracellular detection imaging [15, 16]. Nucleic acids, both DNA and RNA, represent natural polymers and, despite having similar monomeric composition (ATCG in DNA and AUCG in RNA), are very different in folding and biological function. While DNA's primary role is to carry genetic information and the molecule mainly exists as a right-handed double B-form helix, RNA is much more versatile, as this biopolymer can fold into a variety of secondary and tertiary structures allowing it to perform multiple functions in nature

including gene transfer functions (mRNA) [17], adaptor functions (tRNAs) [18–20], guide functions (snRNAs, snoRNAs) [21–23], catalytic functions (ribozymes and the large ribosomal RNA) [24–26], and environmental sensing functions (riboswitches) [27, 28]. It rarely exists just in double-stranded form, which is A-form configuration. Base-stacking and noncanonical base-pairing in RNA provide more room for the design of RNA nanoarchitectures [29].

RNA tertiary structures display a degree of flexibility, which is beneficial for nanofabrication of diverse particles. RNA's flexibility has been well documented by the structural analysis of natural RNA nanomachines such as ribosomes where whole subunits undergo ratchet motions during protein synthesis [30–32], and some individual junctions and internal loops can bend from 180° to 60°, e.g., kink-turn motif [33, 34]. Riboswitches are other natural RNA devices that possess flexibility; upon binding a specific ligand, conformational change occurs that signals for induction or reduction of protein synthesis [27].

Phi29 packaging RNA, or pRNA, forms a hexameric ring that gears the packaging of DNA into a preformed protein capsid [35–37]. An unusually stable three-way junction (3WJ) motif has recently been found within this pRNA molecule [11, 38]. This structural module of the pRNA has attracted scientific attention not only because of its applications to deliver therapeutic moieties to target cells due to its thermostable property, but also as a building block for fabricating stable nanoparticles of different sizes and shapes [39–41]. Detailed analysis of the crystal structure of phi29 pRNA 3WJ motif demonstrated that it is composed of not only Watson–Crick base pairs, but also noncanonical interactions; these include a cis Watson–Crick Watson–Crick (cWW) U–U pair, a base-phosphate (BPh) G–U pair, and two wobble G–U pairs. Additionally, crystal structure analysis revealed that the 3WJ motif was uniquely coordinated by two Mg^{2+} ions that allow stable conformational changes. The 3WJ geometry is a typical A-type [42] and contains three helices: H1, H2, and H3. H1 and H3 form coaxial stacking, aligning along a common axis, and forming ~180° angle. The overall orientation of the motif is planar and helices H1 and H2 form a 60° angle that has been observed in the A-type 3WJs (Fig. 1a). Based on thermodynamic properties and flexibility of the pRNA 3WJ module, we describe a simple method for the construction of various RNA polygons including triangle, square, and pentagon by stretching one of the three 3WJ angles from 60° to 90° and to 108°.

2 Materials

1. PCR reaction kit: Several kits are commercially available; however we use the kit from Promega Corporation, GoTaq® FlexiDNA Polymerase.

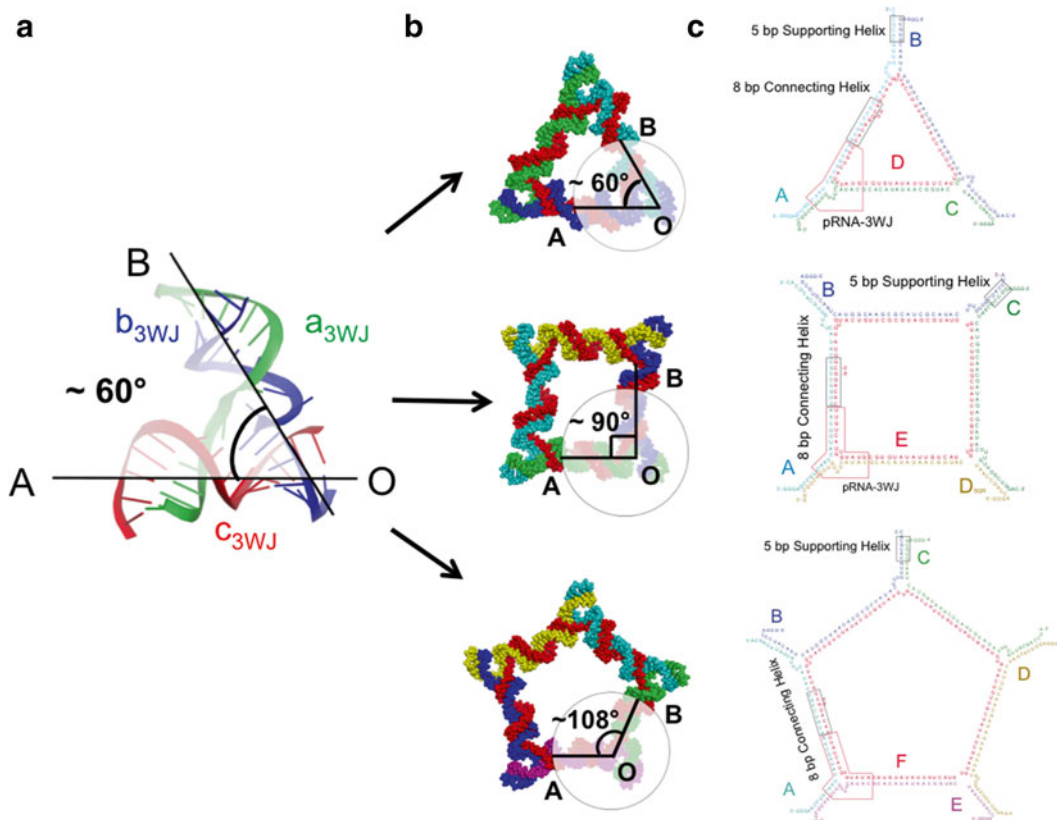


Fig. 1 Structure of pRNA 3WJ and RNA polygons. (a) Crystal structure of pRNA-3WJ showing internal $\angle AOB$ angle used to construct polygons. (b) 3D models of RNA polygons with internal angles highlighted with a *circle*. (c) Sequences and 2D structures of RNA polygons showing connecting helices and supporting helices

2. DNA primers for in vitro RNA transcription dissolved in double-deionized water (dd H_2O) 100 μM stock concentration.
3. QIAquick PCR Purification Kit (<http://www.qiagen.com/>, cat #28706).
4. Ethylenediaminetetraacetic acid (EDTA) 0.5 M, pH = 8.0.
5. Tris-acetate-EDTA (TAE) buffer, pH = 8.0; 1 $\times$ buffer composition: 40 mM Tris base, 20 mM acetic acid, and 1 mM EDTA.
6. Tris-borate EDTA (TBE) buffer, pH = 8.0; 1 $\times$ buffer composition: 89 mM Tris base, 86 mM boric acid, and 2 mM EDTA.
7. Tris-borate-magnesium (TBM) buffer, pH = 8.0; 1 $\times$ buffer composition: 89 mM Tris base, 86 mM boric acid, and 5 mM $MgCl_2$.
8. Sodium acetate 3 M, pH = 6.5.
9. Tris-magnesium saline buffer, pH = 8.0; 1 $\times$ buffer composition: 50 mM Tris-HCl, 100 mM NaCl, 10 mM $MgCl_2$.

10. Urea 8 M in 20 mM TBE buffer pH=8.0.
11. RNA elution buffer: 0.5 M NH₄OAc, 10 mM EDTA, 0.1 % SDS.
12. Denaturing polyacrylamide; stock 20 % solution composition: Acrylamide/*bis*-acrylamide (29:1), 8 M urea, 1× TBE buffer.
13. RNA nucleotide triphosphates (rNTP) 100 mM, adjust pH to 7.5 with NaOH.
14. Fluorine-modified UTP and CTP at 2' hydroxyl ribose (2'-UTP and 2'-CTP).
15. T7 RNA polymerase transcription buffer; 1× buffer composition: 40 mM HEPES-KOH pH=7.5, 24 mM MgCl₂, 2 mM spermidine, 2 mM, 40 mM DTT.
16. 2'-transcription buffer; 1× buffer composition: 40 mM Tris-acetate pH=8.0, 0.5 mM DTT, 1 mM EDTA, 10 mM Mg(OAc)₂, 0.5 mM MnCl₂, 16 mM spermidine.
17. 3 M Sodium acetate solution in ddH₂O.
18. Temperature gradient gel electrophoresis (TGGE) Standard system, Biometra.
19. PAGE Gel Imaging System: Typhoon FLA 7000.

3 Methods

Detailed procedures describing RNA synthesis, purification, self-assembly, and analysis have been detailed previously [10]. Below, we briefly describe some steps of RNA nanoparticle fabrication and comparison of their thermal stabilities. Synthetic DNA molecules coding for the antisense sequence of the desired RNA were purchased from IDT DNA (www.idtdna.com) and amplified by Polymerase Chain Reaction (Promega Corporation) using primers containing the T7 RNA polymerase promoter sequence (5'-TA ATACGACTCACTATA-3').

3.1 DNA Amplification via PCR Reaction

1. Follow the recommended protocol from Promega Co, summarized below.

Protocol 1. PCR reaction setup	
57 µL	ddH ₂ O
20 µL	5× GoTaq Buffer
10 µL	25 mM MgCl ₂
8 µL	2.5 mM dNTPs
2 µL	100 µM FRW primer ^a
2 µL	100 µM REV primer ^b

(continued)

(continued)

1 μL	Go Taq polymerase
$V_{\text{total}} = 100 \mu\text{L}$	

^aFRW—forward DNA primer containing T7 RNA polymerase promoter sequence

^bREV—reverse DNA primer

On a thermal cycler set up the following steps:

Step #1: 95 °C for 5 min.

Step #2: 95 °C for 1 min.

Step #3: 55 °C for 1 min.

Step #4: 72 °C for 1 min.

Step #5: Repeat Steps #2, 3, and 4 25 times.

Step #6: 72 °C for 5 min.

Step #7: 4 °C for 12 h.

2. Purify PCR products using QIAquick PCR Purification Kit; follow the manufacturer’s protocol for purification steps. Usually, DNA concentration is 0.1–0.2 $\mu\text{g}/\mu\text{L}$ after purification.
3. Check approximate length of purified DNA templates on 2 % agarose gel using 1 $\times$ TAE buffer prior to RNA transcription reaction, *see* **Note 1**.

**3.2 RNA and 2' F-U/C
RNA Enzymatic
Synthesis**

1. Reaction mixtures should be prepared at room temperature. There are two protocols given below that have been applied to template DNAs directing transcription from the T7 class III promoter. Protocol #2 summarizes reagent composition for regular RNA transcription reaction. To produce nuclease-resistant 2'-fluoro-modified RNAs we utilize Y639F mutant T7 RNAP and replace pyrimidines (rUTP and rCTP) with the corresponding 2'-fluoro-analogs (<http://www.trilinkbiotech.com/>); please refer to protocol #3 below.

<i>Protocol 2.</i> In vitro RNA transcription by T7 RNA polymerase	
5 μL	ddH ₂ O
10 μL	5 $\times$ Transcription buffer
10 μL	25 mM rNTPs
5 μL	100 mM DTT
10 μL	DNA template
10 μL	T7 RNA polymerase
$V_{\text{total}} = 50 \mu\text{L}$	

Incubate the mixture at 37 °C for 4–6 h.
Transcription can be scaled up using the same ratios.

<i>Protocol 3. In vitro 2'-F-U/C-modified RNA transcription by Y639F RNA polymerase</i>	
5 μ L	ddH ₂ O
5 μ L	10 $\times$ 2'-F-transcription buffer
5 μ L	100 mM DTT
5 μ L	50 mM 2'-F CTP
5 μ L	50 mM 2'-F UTP
5 μ L	50 mM rGTP
5 μ L	50 mM rATP
10 μ L	DNA template
5 μ L	Y639F RNA polymerase
$V_{\text{total}} = 50 \mu\text{L}$	

Incubate the mixture at 37 °C overnight.

Transcription can be scaled up using the same ratios.

2. Terminate transcription reaction by adding 1 μ L of RNase-free DNase I and incubate for additional 30 min to hydrolyze DNA primers.
3. Add the same amount of 8 M urea in TBE to the transcription reaction and run the samples on denaturing PAGE (15 % acrylamide, 29:1 acrylamide:bis-acrylamide, 8 M urea) in 1 $\times$ TBE buffer at 120 V at ambient temperature.
4. Place the gel on a TLC plate and excise the RNA band under UV light. Extract RNA from gel slices using 0.5 mL of RNA elution with 2-h incubation at 37 °C.
5. Precipitate RNA molecules with cold ethanol (2.5:1 (Ethanol:RNA) volume ratio) and 3 M sodium acetate (1:10 volume ratio), rinse twice with cold 80 % ethanol, dry under vacuum, and redissolve in dd H₂O.
6. Measure RNA concentration and store all samples at –20 °C and also, refer to the **Notes 2** and **3** for troubleshoot.

3.3 Stretching the Intra-helical Angle Formed Between H1 and H2

Three-dimensional computer models of the RNA triangle, square, and pentagons were built by manual alignment and adjustments of the pRNA 3WJ junction using Swiss-PDB viewer. For example, the triangle model was constructed by aligning 3 $\times$ 3WJ motifs at the correct angle and distance by “auto-fit” tool or by manual adjustment in Swiss-PDB viewer. H1 was then connected to H2 of each adjacent 3WJ module by double-stranded A-type RNA helix. This “connecting” helix is eight base pairs in length, which results in flat nanoparticles, detailed structure being illustrated in Fig. 1. At each corner, the external helix of the 3WJ is extended by five base pairs as a “supporting” helix to increase stability and specificity among strands. The resulting models contained the 3WJ motif at the

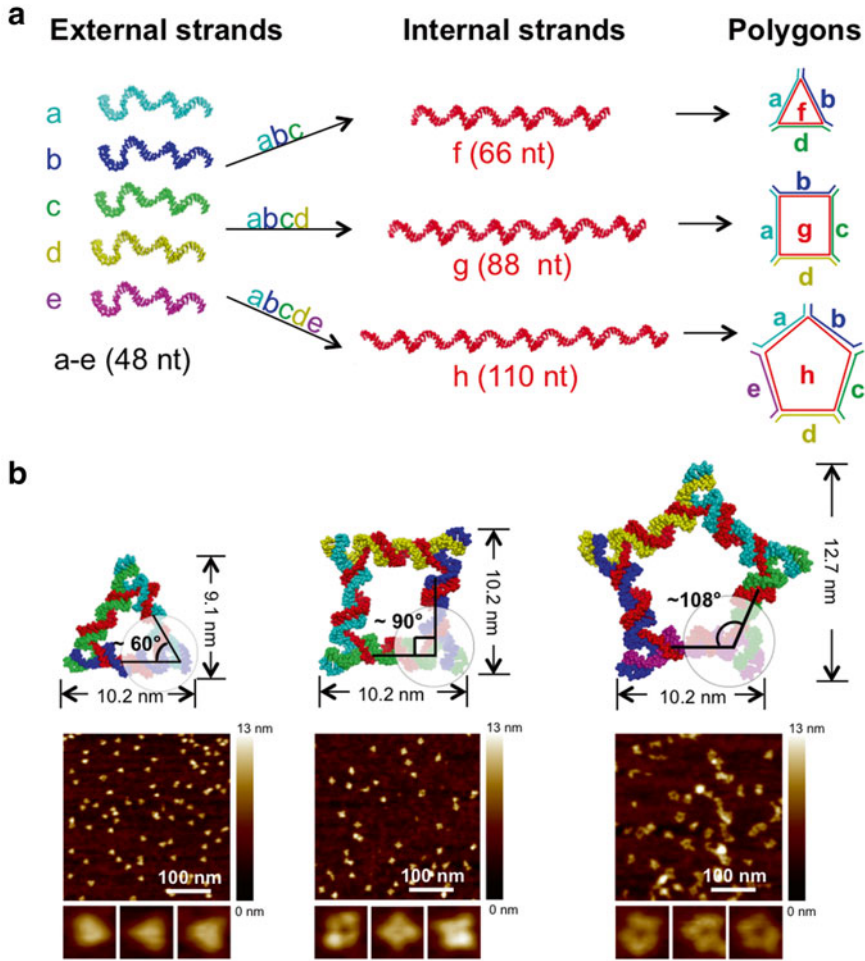


Fig. 2 Construction method of RNA polygons. (a) Combination of external strands with specific internal strand results in corresponding polygons. (b) Annotated size models of RNA polygons and corresponding AFM images are *below* the models

corners with the inner angle of each polygon corresponding to $\angle AOB$ (Fig. 1b). The 3D models are composed of different numbers of RNA strands called short strands (external) and long strands (internal) as shown in Fig. 2a. The number of short strands corresponds to the number of sides of the corresponding polygon, with the internal strand interconnecting all external strands, generating compact structures that are completely double stranded. For example, construction of the triangular nanoparticle consists of three short external RNA strands and one long internal strand, resulting in a 3:1 ratio of short strands to long strands. To build the square nanoparticle this ratio should be 4:1, meaning that there are 4 short strands and 1 long strand; to construct pentagon polygons the ratio is 5:1. Additionally, the long strand for each polygon is incrementally increased by two helical turns, ~22 nucleotides (nt). As such, the long strand for the triangle is 66 nt, the square long strand is

88 nt, and the pentagon long strand is 110 nt. Thus, increasing the number of external strands and the length of the internal strand, the angle on the inter-helical $\angle AOB$ increased from 60° to 90° and 108° allowing for 2D formation of corresponding triangle, square, and pentagon polygons (Fig. 2b). However, this approach is limited as stretching $\angle AOB$ to 120° did not result in the formation of a hexagon (results are not shown). The pRNA 3WJ motif has reached a maximum tension, as it appears that stretching of the 3WJ angle past 108° will not result in stable nanoparticles. The measured widths, from one corner to another, were 10.2 nm, while the heights differed as follows: triangle = 9.1 nm, square = 10.2 nm, and pentagon = 12.7 nm (Fig. 2b).

3.4 Sequence Design and Optimization Prior to RNA Synthesis

The final RNA sequences for each polygon were optimized by *mfold* [43] and *nupack* [44] programs to eliminate any stable secondary structures between short strands, between inappropriate regions of long and short strands, and among individual long strands. Each RNA polygon contained identical sequences in each corner corresponding to pRNA 3WJ $\angle AOB$, which have similar folding energy. The folding energy of the pRNA 3WJ at each corner is believed to be responsible for folding and stability of the polygonal structures. The eight connecting base pairs (bp) and five supporting bp were chosen to be different to prevent nonspecific interactions between short strands. However, the free energy of the duplexes was kept similar, within ± 2 kcal/mol ΔG range, to promote fidelity while folding. The resulting sequences and secondary structures of RNA polygons are demonstrated in Fig. 1c with indication of connecting and supporting nucleotides.

3.5 Polygon Assembly

1. Mix RNA strands corresponding to triangle, square, and pentagon (1 μ M final concentration) in 1 $\times$ TMS, and bring final volume to 10 μ L with dd H₂O (*see* Table 1).
2. Heat the mixture of RNAs to 80 $^\circ$ C for 5 min and slowly cool down to 4 $^\circ$ C over 1 h. Alternatively, the RNA mixture can be incubated for 37 $^\circ$ C for 30 min to achieve self-assembly.
3. Purify the assembled RNA polygons on native PAGE (6 % acrylamide, in 1 $\times$ TBM buffer), and run the gel at 80 V for 3–4 h in a cold room.
4. After the gel run is complete, place the gel on TLC plate and excise the RNA band under UV light. Elute RNA from gel slices using 0.5 mL of RNA elution buffer and 10 mM MgCl₂.
5. Precipitate RNA polygons with cold ethanol (2.5:1 volume ratio) and 3 M sodium acetate (1:10 volume ratio), rinse twice with cold 80 % ethanol, dry under vacuum, and rehydrate in TMS buffer.
6. Measure RNA concentration and store all samples at -20 $^\circ$ C (please refer to the **Note 4** for troubleshoot).

Table 1
RNA polygon assembly setup

Triangle		Square		Pentagon	
1 μ L	10 μ M RNA a	1 μ L	10 μ M RNA a	1 μ L	10 μ M RNA a
1 μ L	10 μ M RNA b	1 μ L	10 μ M RNA b	1 μ L	10 μ M RNA b
1 μ L	10 μ M RNA c	1 μ L	10 μ M RNA c	1 μ L	10 μ M RNA c
1 μ L	10 μ M RNA D	1 μ L	10 μ M RNA d	1 μ L	10 μ M RNA d
1 μ L	10 $\times$ TMS	1 μ L	10 μ M RNA E	1 μ L	10 μ M RNA e
5 μ L	ddH ₂ O	1 μ L	10 $\times$ TMS	1 μ L	10 μ M RNA F
		4 μ L	ddH ₂ O	1 μ L	10 $\times$ TMS
				3 μ L	ddH ₂ O

3.5.1 Polygons' Thermal Stability Comparison

1. After resuspending PAGE-purified polygons in 1 $\times$ TMS dilute the stock solutions to 0.5 μ M concentration.
2. Follow the Biometra protocol (<http://www.biometra.de/>) to cast the gel. Use native 6 % polyacrylamide solution in 1 $\times$ TBM buffer.
3. Run the gel on TGGE using a temperature gradient of 40–80 $^{\circ}$ C applied at constant 100 V for 1 h.
4. After removing the gel from the TGGE instrument, stain with ethidium bromide solution (*see* **Note 5**), and visualize RNA bands using gel imaging system (e.g., Typhoon FLA 700 GE healthcare (<http://www.gelifesciences.com/>)).
5. Using ImageJ software (<http://imagej.nih.gov/ij/>) integrate the intensity of the RNA bands in each well of the gel.
6. Divide the intact nanoparticle intensity (upper most band) by the total intensity of the well to determine nanoparticle formation percentage (example gel is shown in Fig. 3) and plot the data points with temperature on the x -axis and percentage of nanoparticle formation on the y -axis.
7. Fit the data to a nonlinear fitting curve, taking 50 % nanoparticle formation to be the melting temperature of the polygon.

4 Notes

1. PCR:
 - (a) Incorrect size of PCR products might be due to incorrect PCR primer design. Carefully redesign the DNA primers.

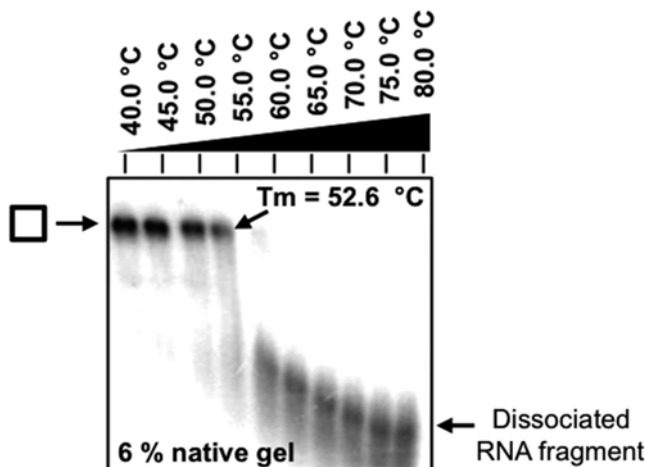


Fig. 3 Representative 6 % TGGE gel of the square RNA polygon melting. The *top bands* represent intact nanoparticle and the downshift is a result of polygon melting

- (b) Nonspecific PCR products are usually due to poor PCR condition setup or the fact that primers are incorrectly designed. Optimize PCR conditions and redesign DNA primers.
2. Transcription:
 - (a) If product yields are low with a protocol that had already been successfully used for the same template, repeat transcription assay once without any alteration on 20 μL scale. Alternatively, test different enzyme batches or enzymes from alternative suppliers.
 - (b) Check that thawed stock solutions, particularly concentrated transcription buffers, do not contain precipitated ingredients.
3. No or low yield of transcription of regular or 2'-F-modified RNA:

This is typically due to impure DNA template. Prepare new template DNA; take particular care to effectively remove salts. Check sequence of T7 promoter at the 5'-end of the forward DNA primer.
4. No or low yield of assembled polygons:
 - (a) Carefully analyze RNA sequence, run secondary structure folding using Mfold, and thoroughly examine assembly conditions.
5. Determining melting temperature of polygons:
 - (a) Ethidium bromide is toxic. Be sure to wear gloves when handling ethidium bromide and only use it in a properly ventilated fume hood.

5 Conclusion

As nanotechnology progresses more challenges are being encountered. One of the most important challenges to overcome is the thermo-stability of RNA nanoparticles. In this chapter we described a simple method to fabricate stable yet functional RNA polygons by stretching the native angle of the RNA three-way junction from phi29 motor pRNA. Polygons that display high yield of formation were achieved via one pot self-assembly of multiple RNA fragments. The key to creating polygons utilizing the same building block is changing the length of the internal RNA strand and increasing numbers of short RNA strands. This induced stretching of the $\angle AOB$ angle of the RNA three-way junction from 60° to 90° and 108° , resulting in self-assembly of elegant RNA triangular, square, and pentagonal nanoparticles. Intermolecular interactions of building blocks within the complex such as kissing loops, receptor loop, or “sticky ends” were avoided by bridging through base pairing between corners of polygons using RNA double helices. Thus, this system is advantageous with increased thermo-stability in the overall construct and yet tunability of the size, and shape of nanoparticles.

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Conflicts of interest: P.G. is a co-founder of Kylin Therapeutics, Inc.; RNA Nano LLC; and Biomotor and RNA Nanotech Development Co., Ltd.

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RNA-Mediated CdS-Based Nanostructures

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Abstract

The colloidal method provides an important tool to synthesize different nanostructures and their assemblies. The coating of colloidal inorganic nanostructures by the biotemplate of similar dimension not only provides them the thermodynamic stability but also imparts the chemical features to grow in different dimensionalities and capabilities for molecular recognition. In this chapter we describe detailed methodology for the synthesis and characterization of CdS nanoparticles templated by ribonucleic acid (RNA) derived from torula yeast. The binding of RNA passivates the surface of CdS nanostructures and controls their optical properties. The presence of excess Cd^{2+} ions induces the folding and polarization in RNA-mediated CdS to enhance the supramolecular interactions among different building blocks. It produces CdS-based nanostructures of varied morphologies in the process of self-assembly. An interaction of the multi-functionalities of RNA with the excess $\text{Cd}^{2+}/\text{Zn}^{2+}$ ions induces the spontaneous folding and polarization in RNA-mediated CdS/ZnS nanostructures and enhances the non-covalent bonding interactions among their building blocks. The self-organization in CdS/ZnS semiconducting nanosystem results in the production of novel tubular morphology.

Key words RNA, CdS, Synthesis, Supramolecular, Nanoparticles, Nanotubes, Nanostructures, Self-assembly

1 Introduction

The base pairing in nucleic acids (DNA/ribonucleic acid (RNA)) has been explored extensively to design 3D nanoarchitectures of novel biomaterials in the process of self-assembly [1–3]. Unlike DNA, RNA scaffolds are largely present with single strandedness and provide extended flexibility and spatial organization [4, 5]. RNA helix generally displays “A” type of configuration, which is more stable than “B” form of DNA helix based on the estimation of $-G^0$ helix [5, 6]. RNA-mediated nanostructures are thus expected to be thermodynamically more stable as compared to those of templated by DNA. Among three types of cellular RNA molecules (ribosomal RNA (rRNA), messenger RNA (mRNA),

and transfer RNA (tRNA)), rRNA is the largest (~20 nm) and tRNA (~5 nm) the smallest [7, 8]. All these characteristics of RNA make it a convenient tool to design functional materials with varied size, shapes, and dimensionalities.

Alkali, alkaline, and transition metal ions are known to bind RNA strands to influence its folding. The negative charge on RNA will allow the binding of different metal ions to influence its three-dimensional architecture to cause simultaneously folding and stabilization [9, 10]. A difference in the binding strength of different cations and the stability of their complex with RNA, the inter- and intra-strand assembling properties of RNA in the presence of metal cations together with its propensity to fold into various secondary and tertiary structures could thus be utilized to stabilize and organize various semiconductor and metal NPs into specific shapes of predefined morphologies and size [11–16]. Such interactions would result in the reduction of the surface energy of nanomaterials to prevent clustering and aggregation besides imparting them with the large functional and structural diversity and characteristic properties. This allows fabricating 1D, 2D, and 3D self-assembled RNA-semiconducting/metal nanosystems with varied morphologies and engineered physicochemical properties.

In recent years biotemplating has emerged as an important tool to fabricate new materials with enhanced physicochemical properties besides introducing the biocompatibility [17]. RNA having nanodimension, long-range hierarchical order, and large functionalities (Fig. 1) makes it an attractive template for synthesizing RNA-mediated building blocks of nanosized colloidal CdS. In a previous report different sequences of RNA (wild-type (WT) tRNA

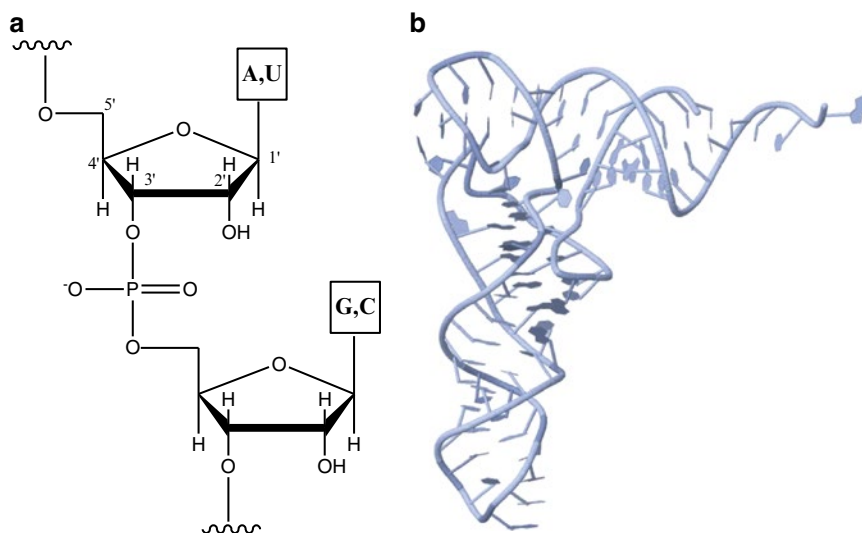


Fig. 1 Molecular structure of RNA (a); 3-Dimensional structure of tRNA (b) (image created using Jmol from PDB 1 ehz)

and (MT) tRNA) were employed for the stabilization of CdS nanoparticles [8]. In this chapter, we explain the detailed synthetic protocol for CdS nanoparticles and their self-assembly mediated by RNA extracted from torula yeast. These nanostructures have been characterized by employing various spectroscopic and microscopic techniques. Among different building blocks the non-covalent supramolecular interactions involving RNA-RNA and RNA-CdS functionalities allow the formation of various supernanostructures. The presence of excess Cd^{2+} ions induces a folding of RNA-mediated CdS to change the shapes of CdS nanostructures in the process of self-assembly.

2 Materials

Prepare all solutions in either ultrapure water with the conductivity at $18 \text{ M}\Omega$ at 25°C or in double-distilled water. For the preparation of double-distilled water, we transfer single-distilled water to an electrically heated 5 L Borosil glass distillation apparatus. Appropriate amounts of sodium hydroxide and potassium permanganate were added to this solution. Sodium hydroxide maintained the desired conductivity of the solution while KMnO_4 oxidized the traces of organic impurities.

All solutions were prepared freshly before each study in ultrapure/double-distilled water since the as-synthesized samples are very sensitive to impurities. All glassware were cleaned thoroughly by chromic acid/detergent and then rinsed with ordinary water followed by distilled water at least three times and dried in oven prior to use.

2.1 Reagents, Glassware, and Other Components

1. Cadmium perchlorate.
2. Zinc acetate.
3. RNA (type VI) (is a heterogeneous mixture of RNA molecules with varied molecular weights and was derived from torula yeast. It contained phosphorous contents of $\sim 8.5\%$).
4. Sulfuric acid.
5. Hydrochloric acid.
6. Nitric acid.
7. Ethanol.
8. Potassium dichromate.
9. Oxalic acid.
10. Perchloric acid.
11. Acridine orange.
12. Ferrous sulfide.
13. NaOH.

14. Potassium permanganate.
15. Nitrogen with purity >99.9 % (Sigma, India) for deoxygenating the samples during synthesis (*see* **Note 1**).
16. Measuring flasks, conical flasks, and graduated test tubes of Pyrex or Borosil glass of different capacities.
17. Quartz cuvettes of 10, 5, 4, and 2 mm path lengths.
18. Carbon-coated copper grids.
19. Glass slides.

2.2 Equipment

A description of the make and model of analytical techniques employed for the characterization of the reported nanostructures is provided below:

1. *Spectrophotometer*

The UV-visible absorption spectra were recorded on a Shimadzu UV-2100S spectrophotometer.

2. *Spectrofluorophotometer*

Steady-state excitation and emission spectra were measured on a Shimadzu RF-5301-PC spectrofluorophotometer fitted with two monochromators, one each in excitation and emission port. To ensure the prevention of undesired radiations suitable glass filters were also mounted in both excitation and emission ports.

3. *Atomic Force Microscopy (AFM)*

The surface topographic analysis of the samples was performed on a NTEGRA (NTMDT) atomic force microscope. It has a built-in optical system which allows imaging of the scanning process with 1 μm resolution. The resolutions in x - y and z directions are 0.1–1.0 nm and 0.04 nm, respectively.

4. *Transmission Electron Microscope (TEM) with Selected Area Electron Diffraction (SAED)*

Electron microscopy and SAED measurements were performed on a Fei-Philips Morgagni 268D and FEI-TECNAI digital TEM at accelerating voltages of 100 kV and 200 kV, and magnifications up to 280,000 $\times$ and 1,100,000 $\times$, respectively. Size of different nanostructures was determined from their TEM micrographs using ImageJ digital micrograph software from NIH, USA.

5. *Field Emission Scanning Electron Microscope (FESEM) Coupled with Energy-Dispersive X-Ray Analysis (EDAX)*

The surface morphology of the samples was analyzed by FEI-QUANTA 200 F field emission scanning electron microscope having accelerating voltage range from 200 V to 30 kV. The elemental analyses of the synthesized materials were performed using EDAX accessory equipped with CCD camera.

6. *X-Ray Diffractometer*

X-ray diffraction patterns were recorded on a Philips DW 1140/90 X-ray diffractometer using Cu K α line (1.5418 Å) of the X-ray source.

7. *NMR Spectrometer*

The ^1H and ^{31}P NMR spectra were recorded on a 500 MHz Bruker Avance 500 Spectrometer. The NMR spectra were plotted by using the Topspin software provided by Bruker.

8. *Infrared Spectrometer*

IR spectra were obtained on a Thermo Nicolet Nexus FTIR spectrophotometer in mid-IR range in KBr medium. All spectra were recorded in transmission mode.

9. *Time-Correlated Single-Photon Counting (TCSPC) Spectrometer*

Fluorescence lifetime and anisotropy decay curves were recorded on a Horiba Jobin Yvon-IBH “FluoroCube Fluorescence Lifetime System” in time domain mode. NanoLEDs and LDs were used as pulsed excitation source working at 1 MHz repetition rate in reverse mode. For samples having higher lifetime, i.e. in the range of microsecond(s), measurements were made in forward mode at lower repetition rate (250 kHz) of pulsed excitation source(s). Automated polarization accessory (Model 5000U-02) was used for anisotropic measurements. The emitted photons in UV-visible range were detected by using thermoelectrically cooled TBX-04-D detector. Decay curves were analyzed by iterative reconvolution technique using multiexponential fitting program provided from IBH. Data analysis was carried out using DAS 6.1 software.

10. *Rotavapor*

The colloidal solution(s) of the semiconductor(s) were solidified on Buchi R-114 and Yamato RE 300 rotavapor(s). The latter was fitted with neocool circulator (CF300).

3 Methods

3.1 Synthesis of RNA-Templated CdS-Based Nanoparticles and Their Self-Assembly

1. Dissolve required amount of RNA (e.g., 0.015 g) in 100 mL water at pH 9.2 under constant stirring and store this RNA solution overnight at 4 °C.
2. Prepare stock solution of $\text{Cd}(\text{ClO}_4)_2$ (0.1 mol/dm 3) in water (*see Note 2*).
3. Prepare H_2S gas by the acid decomposition of FeS. Clean it by passing through the water jacket for removing any water-soluble impurities.
4. Prepare fresh HS^- solution by passing H_2S into a 0.05 M NaOH solution at room temperature till the pH of this solution

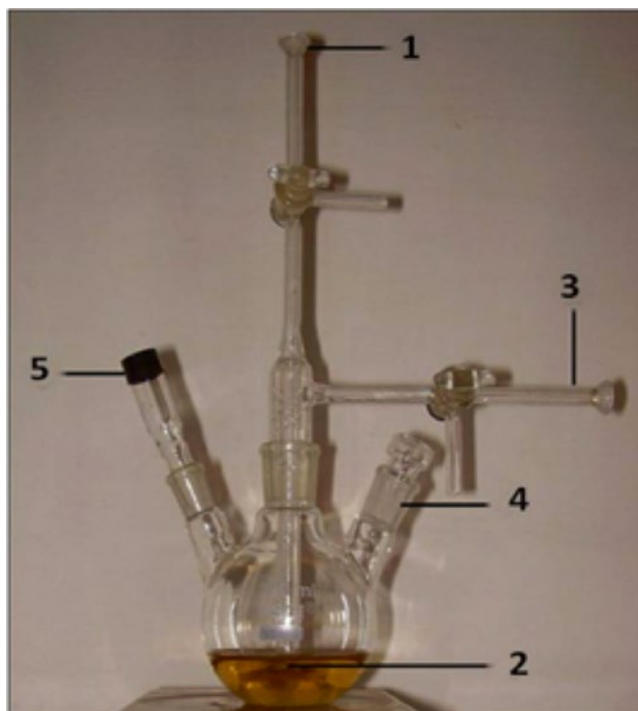


Fig. 2 Glass assembly used for preparing RNA-capped CdS and CdS/ZnS nano-system: (1) Gas inlet; (2) glass frit; (3) gas outlet/deaeration; (4) joint for holding electrode; (5) joint for holding rubber septum

is reduced to about 7, which corresponds to the pK_1 of H_2S (*see Note 3*).

5. For the synthesis of RNA-templated CdS, a three-neck flask equipped with three standard joints was used. The central joint (B-24) was used for holding the bubbler. The bubbler contained the gas inlet tube (1) fitted with glass frit (2) at the bottom for effective flushing nitrogen in the vessel and a gas outlet/deaeration arm (3). The other two side joints were used for holding pH electrode (B-19) (4) and rubber septum (B-14) (5) (Fig. 2).
6. Add required volume (20–60 μL) of 0.1 mol/dm^3 stock solution of Cd^{2+} (to get the final concentration of Cd^{2+} from 2×10^{-4} to 6×10^{-4} mol/dm^3) to RNA solution containing desired amount of RNA (0.01–0.2 mg/mL) in 100 mL three-neck flask at pH 9.2.
7. Digest RNA- Cd^{2+} solution overnight at 4 °C (*see Note 4*).
8. Purge N_2 continuously under constant stirring for its deoxygenation at pH 9.2 (*see Note 5*).

9. Inject 200 μL of freshly prepared solution of HS^- (0.05 mol/dm^3) into the deaerated aqueous solution of RNA- Cd^{2+} at its desired concentration adjusting the final concentration of HS^- to $1 \times 10^{-4} \text{ mol/dm}^3$. The colorless RNA- Cd^{2+} solution immediately turned yellow resulting in the formation of transparent colloidal solution of CdS (*see Note 6*).
10. Flush CdS solution again with N_2 for about 10 min.
11. Adjust the pH of the solution at 9.2 by using dilute NaOH and perchloric acid solution (*see Note 7*).
12. Various self-assembled colloidal nanostructures of RNA-CdS were grown by aging of RNA-CdS for different durations (days) (*see Notes 8 and 9*).

3.1.1 Optical Absorption and Fluorescence Measurements

1. Record the absorption spectra of RNA-CdS in visible (250–800 nm) in two-side frosted quartz cuvette with 10/5 mm path length (*see Note 10*).
2. Record the fluorescence spectra of RNA-CdS in visible (400–800 nm) in all-side clear/transparent quartz cuvette with 10/5 mm path length (*see Note 11*).
3. Change the amount of RNA (0.001–0.015 g/mL) and Cd/S ratio (2–6).
4. Record absorption and fluorescence spectra of the synthesized samples under varied experimental conditions. An example of absorption and emission of RNA-CdS is shown in Fig. 3.
5. Age the colloidal solution of CdS containing Cd/S ratio 4 for a period of 2 months at 4 °C. Record their absorption and fluorescence spectra (*see ref. 12*).

3.1.2 TEM Analysis

1. Apply a drop of colloidal RNA-CdS solution onto copper-coated TEM grid. Wipe out the excess solution with filter paper.
2. Dry TEM grid in the dark in desiccators at room temperature.
3. Record TEM micrographs of fresh and aged samples to obtain good images (Fig. 4).

3.1.3 Charge Carrier Dynamics

1. Record the time-resolved fluorescence decay of different samples in quartz cuvette of varied thickness (*see Notes 10 and 11*).
2. Record the fluorescence decay kinetics using photon counting technique in reverse mode.
3. An example of charge carrier dynamics of RNA-CdS containing varied Cd/S (*see ref. 12*) is shown in Fig. 5 (*see Note 12*).

The structural information about CdS in the colloidal sample(s) could be obtained by employing XRD (*see Notes 13 and 14*).

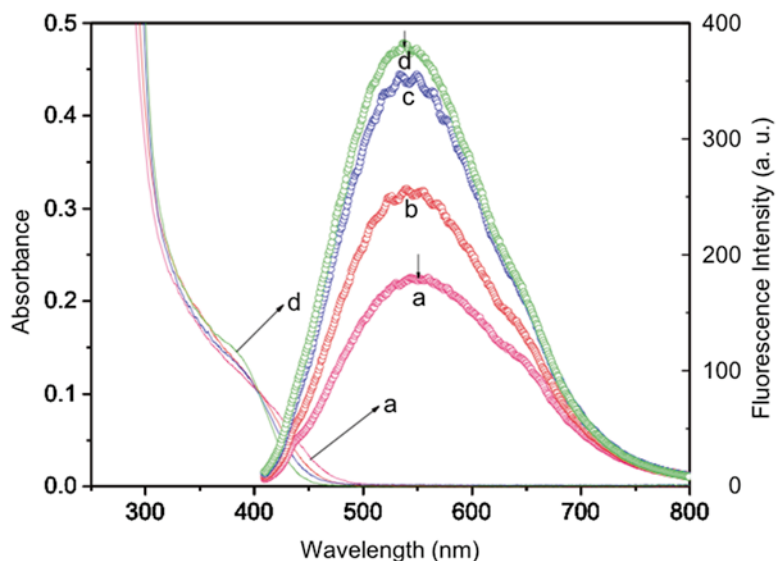


Fig. 3 Effect of [RNA] (g/100 mL), 0.001 (*a*); 0.003 (*b*); 0.010 (*c*); 0.015 (*d*), on the electronic and fluorescence spectra of CdS at pH 9.2 ($\lambda_{\text{ex}} = 380$ nm). An increase in [RNA] (0.001–0.015 g/100 mL) regularly blue shifted the fluorescence maxima from 552 nm (2.25 eV) to 530 nm (2.34 eV) with an enhancement in the fluorescence intensity. For a typical 0.015 g/100 mL of RNA, the fluorescence intensity is increased by twofold to that observed for 0.001 g/100 mL of RNA. Thereafter, any increase in RNA resulted in a decrease in the fluorescence intensity (*see ref. 12*). Reprinted from *ref. 12*. Copyright 2008 American Chemical Society

Interactions of different moieties of RNA with Cd^{2+} and CdS may be analyzed by using IR and NMR spectroscopic techniques (*see Notes 15 and 16*). The morphologies of RNA-CdS self-assemblies exhibit the dependence on the used experimental conditions (*see Notes 17 and 18*) (*see ref. 12*).

3.2 Synthesis of RNA-Templated CdS/ZnS Nanotubes

1. Prepare stock solution of $\text{Zn}(\text{CH}_3\text{COO})_2$ (0.1 mol/dm^3) in water. Solution of RNA, $\text{Cd}(\text{ClO}_4)_2$, and HS^- were prepared as described under Subheading 3.1.
2. Mix required volumes of 0.1 mol/dm^3 stock of Cd^{2+} and Zn^{2+} ($20 \text{ }\mu\text{L}$ each) with RNA solution ($0.07\text{--}0.2 \text{ mg/mL}$) in 100 mL three-neck flask to adjust the final concentration of Cd^{2+} and Zn^{2+} at $2 \times 10^{-4} \text{ mol/dm}^3$ (shown in Subheading 3.1, Fig. 2) and pH 9.2.
3. Purge RNA- $\text{Cd}^{2+}/\text{Zn}^{2+}$ solution continuously with N_2 under constant stirring for 15 min for its deoxygenation. Adjust the pH of the solution to 9.2.
4. Inject $500 \text{ }\mu\text{L}$ of freshly prepared solution of HS^- (0.5 mol/dm^3) into the above deaerated aqueous solution of RNA- $\text{Cd}^{2+}/\text{Zn}^{2+}$ and adjust the final concentration of HS^- to $1 \times 10^{-4} \text{ mol/dm}^3$.

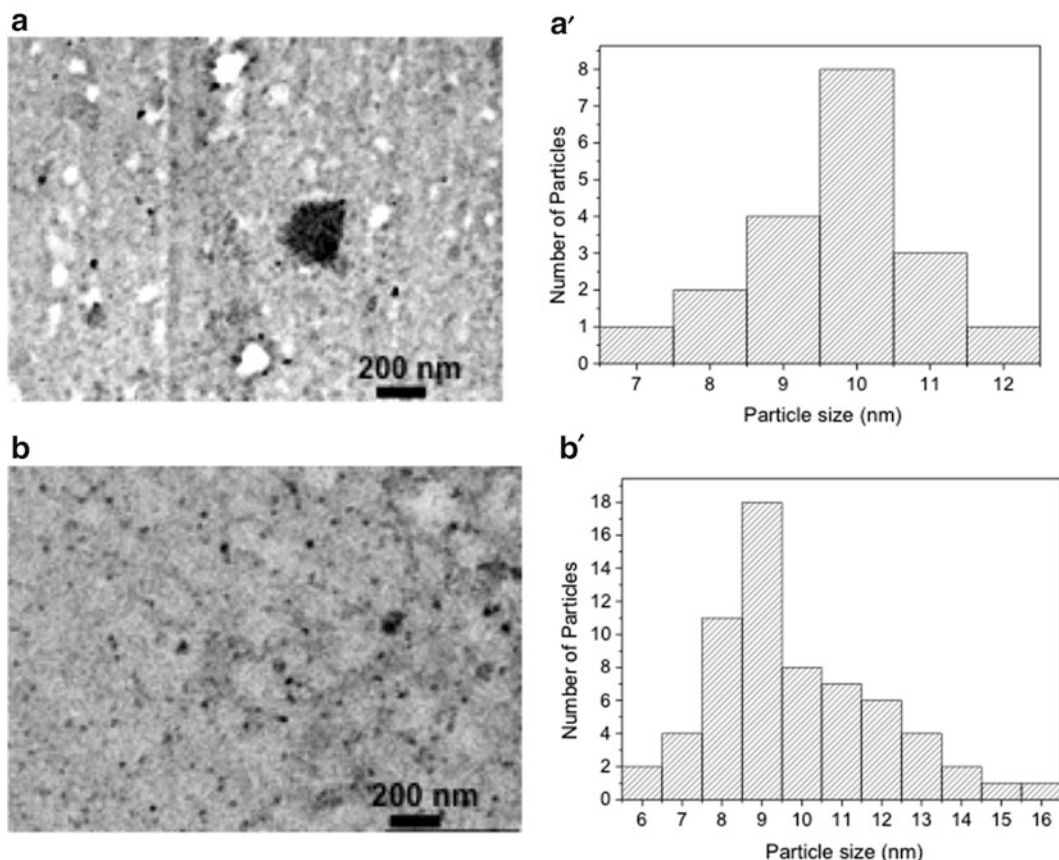


Fig. 4 TEM images of RNA-mediated CdS with Cd/S ratio 4 depicting the effect of 2-month aging, fresh (**a**, **a'**) and aged (**b**, **b'**), on the particle size and size distribution. Aging for 2 months reduced the average size of nanoparticles from 10 to 9 nm. Average size of nanoparticles strongly depends on experimental conditions, e.g., [RNA], Cd/S, and duration of aging (*see ref. 12*). Reprinted from ref. 12. Copyright 2008 American Chemical Society

5. Flush N_2 again for about 10 min.
6. Add 70 μL of 0.1 mol/dm^3 of Zn^{2+} to the colloidal solution in order to adjust the $[\text{Zn}^{2+}]$ at $7 \times 10^{-4} \text{ mol/dm}^3$.
7. Flush again with N_2 for 15 min and store at 4°C in the dark for characterizations.
8. Maintain the pH of the solution to 9.2 at each stage.

3.2.1 Optical Absorption and Fluorescence Measurements

1. Record the absorption spectra of RNA-CdS/ZnS in UV-visible (250–800 nm) in two-side frosted quartz cuvette with 10/5 mm path length.
2. Record the fluorescence spectra of RNA-CdS/ZnS in visible (400–700 nm) in all-side clear/transparent quartz cuvette with 10/5 mm path length.

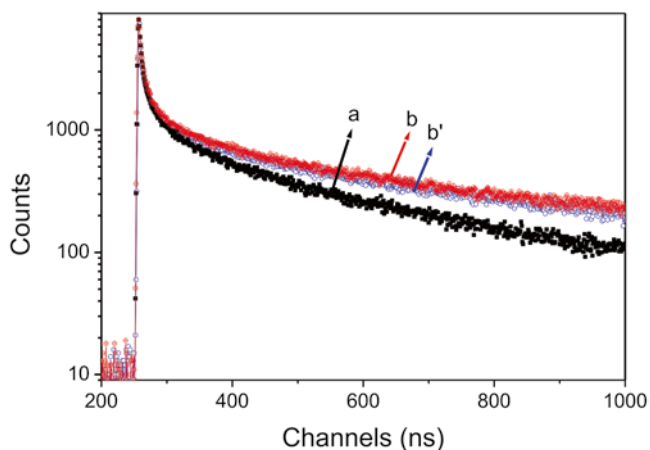


Fig. 5 Fluorescence decay curves of CdS having Cd/S molar ratio 2: fresh (a); 4: fresh (b), aged (b'). [$\lambda_{\text{ex}}=375$ nm; $\lambda_{\text{em}}=530$ nm]. Reprinted from ref. 12. Copyright 2008 American Chemical Society

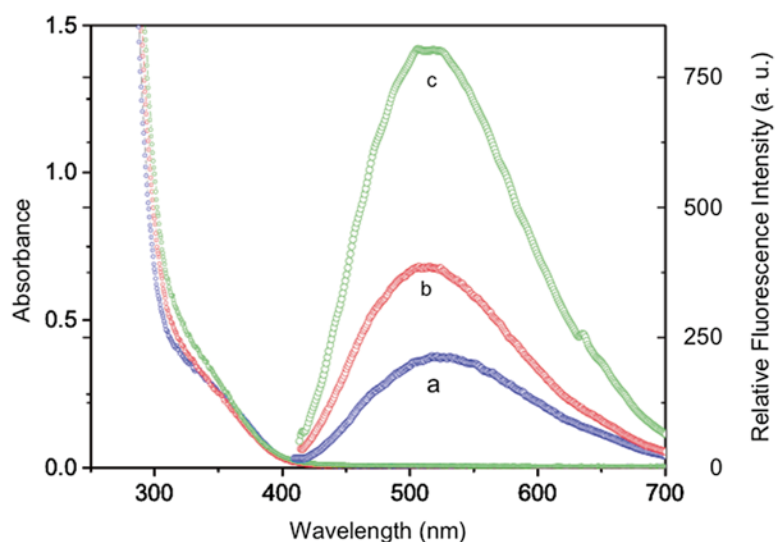


Fig. 6 Electronic and fluorescence spectra of RNA-CdS/ZnS co-colloids containing [RNA] = 0.015 g/100 mL; (10^{-4} mol/dm³) [Cd^{2+}] = 2; [Zn^{2+}] = 2; and [HS^-] = 2.5 having varied amount of Zn^{2+} (10^{-4} mol/dm³): 0 (a); 3 (b); 7 (c), [$\lambda_{\text{ex}}=400$ nm]. An increase in [Zn^{2+}] from 1×10^{-4} to 7×10^{-4} mol/dm³ exhibited a regular red shift in the onset of absorption and excitonic peak. The fluorescence maximum is blue shifted from 520 nm (2.38 eV) to 509 nm (2.44 eV) and the intensity of this band is enhanced by a factor of about 3.7. Reprinted from ref. 14. Copyright 2009 Royal Society of Chemistry

3. Prepare emission specimen by changing the amount of RNA (0.001–0.015 g/mL) and Cd/S ratio (2–6).
4. Record the absorption and fluorescence spectra of the samples (Fig. 6).

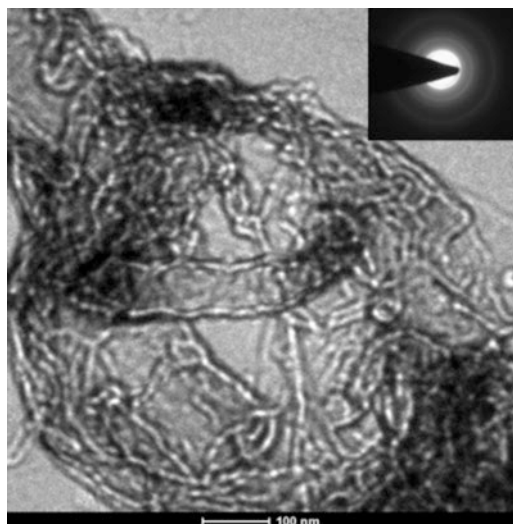


Fig. 7 TEM image of RNA-CdS/ZnS depicting the formation of networks of nanotubes: having an average diameter of 18 nm with an inner diameter of 10 nm and a wall thickness of about 4 nm. *Inset*: Its SAED pattern exhibiting the diffused rings. Reprinted from ref. 14. Copyright 2009 Royal Society of Chemistry.

3.2.2 TEM and SAED Analysis

1. Prepare sample for TEM and SAED analysis as described in Subheading 3.1.2.
2. An example of TEM and SAED of RNA-CdS/ZnS nanotubular structures is shown in Fig. 7.

3.2.3 Charge Carrier Dynamics

1. Record the relaxation kinetics of colloidal RNA-mediated CdS/ZnS containing excess Zn^{2+} ($7 \times 10^{-4} \text{ mol/dm}^3$) (SP1) and in the absence of excess Zn^{2+} (SB) at pH 9.2.
2. Measure the fluorescence decay at 509 nm (2.44 eV) using the 405 nm (3.06 eV) excitation wavelength (Fig. 8).
3. Calculate the average lifetime following three-exponential decay kinetics.

3.2.4 Fluorescence Anisotropy

1. Record the fluorescence anisotropy of the samples SP1 and SB by using excitation wavelength of 405 nm (3.06 eV) excitation wavelength and measuring the emission at 509 nm (2.44 eV) employing polarizers.
2. An analysis of the rotational correlation times is shown in Table 1. Compare the values of θ_1 and θ_2 .

The structural information about CdS, ZnS, and other species of precursors in the colloidal sample(s) could be obtained by employing XRD (*see Note 19*). The interactions between different moieties of RNA with the precursor ions and CdS/ZnS may be analyzed by using IR spectroscopy (*see Note 20*).

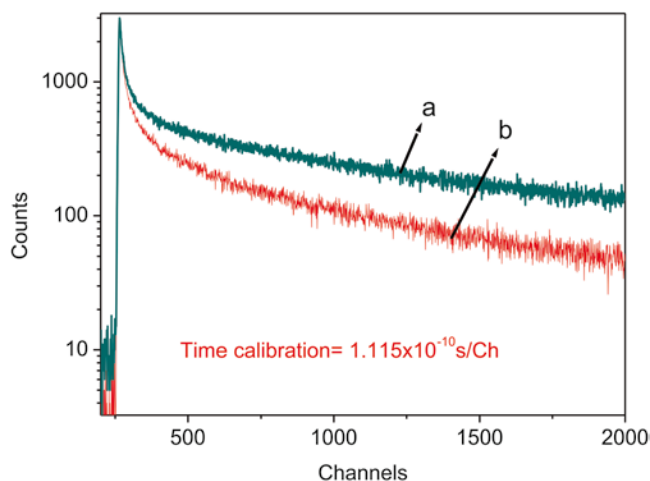


Fig. 8 Fluorescence decay curves of SP1 (a) and SB (b). [$\lambda_{\text{ex}} = 405$ nm; $\lambda_{\text{em}} = 509$ nm]. The $\langle \tau \rangle$ for RNA-CdS/ZnS containing excess Zn^{2+} and without excess Zn^{2+} were found to be 70 ns and 41 ns, respectively (see ref. 14). Reprinted from ref. 14. Copyright 2009 Royal Society of Chemistry

Table 1

Analysis of fluorescence anisotropy decay curves of RNA-CdS/ZnS with and without excess Zn^{2+} at pH = 9.2

RNA-CdS/ZnS	Rotational correlation times (ns)				
	Component 1		Component 2		
	θ_1	Emission %	θ_2	Emission %	χ^2
With excess Zn^{2+} (SPI)	2.2 (0.1556)	57.8	52.0 (0.1134)	42.2	1.0
Without excess Zn^{2+} (SB)	2.9 (0.0959)	42.9	25.0 (0.1275)	57.1	1.0

[$\lambda_{\text{ex}} = 405$ nm; $\lambda_{\text{em}} = 509$ nm]. Reprinted from ref. 14. Copyright 2009 Royal Society of Chemistry (θ = rotational correlation time)

The morphologies of RNA-CdS/ZnS self-assemblies could also be examined by AFM and FESEM (see Notes 21 and 22) (see ref. 14).

4 Notes

1. All other chemicals are of analytical grade and were used without any further purification.
2. Cd^{2+} is toxic; due care has to be exercised during the preparation of the solution to avoid any contact with cadmium.

3. H₂S gas is highly poisonous. Therefore, its preparation must be carried out in the fume hood. Direct inhalation of the gas should be avoided.
4. For better fluorescence properties, RNA-Cd²⁺ should be digested overnight at 4 °C prior to colloidal synthesis.
5. It is very important to deoxygenate the solution before the addition of HS⁻.
6. Colloidal CdS solution/solid is photoactive and should be stored and handled in the dark.
7. The pH meter was calibrated manually by using buffers of pH 4 (potassium hydrogen phthalate) and 9.2 (borax). Thereafter, the glass electrode was left overnight dipped in dilute HCl solution.
8. The aging of colloidal RNA-CdS solution should be carried out in the dark at 4 °C in a tightly closed vessel to avoid its air oxidation as well as any change in concentration through evaporation.
9. The growth of nanostructures can be controlled by the judicious selection of experimental conditions such as [RNA], Cd/S, pH, and period of aging.
10. For samples having higher absorption coefficient, 4 or 2 mm quartz cuvettes are used in order to avoid the inner filter effect.
11. To ensure the prevention of undesired radiations suitable glass filters were also mounted in both excitation (300 nm) and emission (400 nm) ports.
12. The average lifetime ($\langle \tau \rangle$) was calculated using the following relationship [18]:

$$\langle \tau \rangle = \frac{\sum_{i=1}^n b_i \tau_i^2}{\sum_{i=1}^n b_i \tau_i}$$

where b_i is the exponential factor corresponding to the respective lifetime component τ_i ($i = 1$ to n).

13. For XRD and FTIR analysis solid samples were obtained by drying different solutions of precursors and the colloidal solution of RNA-CdS under vacuum using rotavapor at room temperature.
14. The X-ray diffraction patterns of solid samples were recorded by using Cu K α line (1.5418 Å) of the X-ray source. The acceleration voltage was kept at 40 kV with 30 mA flux. For all samples the diffraction patterns were recorded in 2 θ range of 10–100° at a scanning speed of 0.5°/min. XRD patterns for different samples of RNA-capped CdS containing varied ratio of Cd/S (4 and 6) show that CdS is produced in the hexagonal phase.

The broadening of the diffraction pattern at low Cd/S=4 also suggests that the CdS sample prepared under these conditions is amorphous and the particles produced may be fairly small.

15. FTIR spectra of different samples in mid-IR range are recorded in transmission mode by preparing the thin film of the respective solid samples of RNA, RNA-Cd²⁺, and RNA-CdS in KBr medium. An analysis of IR spectra of RNA, RNA-Cd²⁺, and RNA-CdS reveals that all vibrations due to purines and pyrimidine bases, specifically the band(s) due to U, G, A, and C in RNA, depict an interaction with Cd²⁺ and CdS. Thus it is arrived that CdS on RNA matrix is bound to purine, pyrimidine, and the sugar moiety of RNA through Cd²⁺.
16. ¹H NMR of the samples was recorded in 10 % D₂O. NMR spectra of samples were collected in deuterated aqueous solutions at 500 MHz frequency with 32 scans. BBO probe is used to acquire ¹H NMR signals. NMR spectra exhibit all the characteristic features of RNA. Shift in the peak position of RNA upon interaction with Cd²⁺ and CdS was used to assess the interaction of different functionalities of RNA with CdS.
17. For FESEM analysis samples were prepared by applying a drop of colloidal solution on a glass plate. Conductive coating of samples with gold was carried out using ion sputtering prior to imaging. A thin layer of gold should be used to prevent its interference with the samples during imaging. Silver paste is used to make effective ground path for FESEM specimens. FESEM images depicted the formation of different self-assembled nanostructures with varied topology and dimensions under different experimental conditions ([RNA], ratio of Cd/S, and duration of aging). For example at Cd/S=4, FESEM images show formation of network of nanowires with a diameter of about 250 nm after 2-month aging. EDAX analysis of a portion of this nanowire depicts a homogeneous distribution of Cd and S all along the wire.
18. For surface topography analysis of the nanostructures, two- and three-dimensional AFM images of fresh and aged samples are recorded. The samples for AFM imaging were prepared by applying a drop of colloidal solution onto a glass plate and then drying it in desiccators at room temperature in the dark. All the samples were imaged in semi-contact mode. In general fresh samples depicted these particles to be spherical.

However, an increase in molar ratio of Cd/S from 2 to 4 increases the maximum surface roughness of these particles from 4 to 14 nm. The aging of the sample having molar ratio of Cd/S=4 for about 2 months resulted in the reduction of their maximum surface roughness from 14 to 3.5 nm and these particles displayed an organized structure.

19. Structural properties of the as-prepared colloidal solution of RNA-CdS/ZnS containing 7×10^{-4} mol/dm³ of excess Zn²⁺ (SP1) were further examined by recording the XRD patterns of its powder sample. The d-spacing values of RNA-templated CdS/ZnS co-colloids show that CdS, ZnS, and Zn(OH)₂ were produced in hexagonal, wurtzite, and orthorhombic phases, respectively. To further analyze the structure of metal hydroxides, in a control experiment, XRD analysis of the sample obtained by mixing of 2×10^{-4} mol/dm³ Cd²⁺ and 9×10^{-4} mol/dm³ Zn²⁺ at pH 9.2 (R2) was performed. It exhibits the formation of Zn(OH)₂ and Cd(OH)₂ in orthorhombic and hexagonal phases, respectively, under these experimental conditions similar to that observed in XRD pattern of SP1 [14].
20. In order to analyze the interaction of different functional groups in RNA with metal ions and semiconductor nanoparticles, IR spectra of pure RNA, RNA in the presence of Zn²⁺/Cd²⁺ (R2), and SP1 were recorded in the mid-IR range. In the IR spectrum of R2 significant changes in the absorption frequencies due to its various functional groups/moieties compared to that of RNA suggest their interaction with Cd²⁺/Zn²⁺. In the IR spectrum of SP1 a further shift in the energy of absorption band due to A, U, G, and C; PO₂⁻ and 2'-OH in RNA is observed compared to those of pure RNA/RNA in the presence of Zn²⁺/Cd²⁺.
21. Three-dimensional AFM images of the colloidal SP1 depicted the formation of nanotubes of micrometer length (≤ 1 μ m) with a height ranging from 7 to 20 nm. 3D-AFM image of one such isolated tubular structure exhibits the height of the tube of about 18 nm with the surface roughness distribution ranging from 10 to 25 nm.
22. Surface morphology of SP1 was also examined by FESEM. The FESEM image depicts the formation of entangled tubular nanostructure(s) in which nanotubes manifest in the form of bundles of varied dimensions. Aging of this sample for about 2 months induces the aggregation of these nanotubes to form bundles of tubes of larger size. EDAX analysis of this sample depicted the homogeneous distribution of Cd, Zn, and S.

Acknowledgements

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Chapter 17

An Effective Method for Specific Gene Silencing in *Escherichia coli* Using Artificial Small RNA

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Abstract

Knockdown or silencing of a specific gene presents a powerful strategy for elucidating gene function in a variety of organisms. To date, efficient silencing methods have been established in eukaryotes, but not bacteria. In this chapter, an efficient and versatile gene silencing method using artificial small RNA (afsRNA) is described. For this purpose, target-recognizing sequences were introduced in specially designed RNA scaffolds to exist as single-stranded stretches in afsRNA. The translation initiation region of target genes was used as the sequence for afsRNA recognition, based on the theory that this site is usually highly accessible to ribosomes, and therefore, possibly, afsRNA.

Two genes transcribed as monocistrons were tested with our protocol. Both genes were effectively silenced by their cognate afsRNAs.

Key words Artificial small RNA (afsRNA), Gene silencing, Gene knockdown, Target recognition sequence, Single-strandedness, Translation initiation region

1 Introduction

Bacterial small noncoding RNAs (sRNAs) play diverse roles as gene expression regulators in a variety of physiological processes [1–3]. Generally, bacterial sRNAs modulate translation activity and/or endogenous stability of their target mRNAs via RNA-RNA base pairing. In *Escherichia coli* (*E. coli*), about a hundred species of sRNAs have been experimentally identified, and nearly half of these functionally analyzed. The majority of identified sRNAs are encoded *in trans* at different chromosomal locations from target genes, and usually suppress target gene expression by inhibiting translation and inducing mRNA degradation with the aid of an abundant RNA chaperone protein, Hfq. Other sRNAs are encoded *in cis* on strands opposite their target. Unlike *trans*-encoded sRNAs, *cis*-encoded sRNAs have extensive regions capable of base pairing, but the actual base-pairing regions appear limited to

specific sequences. Additionally, their functions are generally independent of Hfq [4, 5].

Several researchers have attempted to design strategies for silencing or knockdown of specific genes in bacteria by mimicking the gene-silencing effects of sRNAs [6–9]. In eukaryotes, induction of specific gene silencing by short interfering RNAs (siRNAs) has been extensively used as a powerful biological strategy for elucidating gene function and developing therapeutic agents [10–12]. Therefore, gene silencing could be achieved using artificial sRNA (afsRNA) loaded with defined target recognition sequences. To design effective afsRNAs in *E. coli*, factors that affect interactions with target mRNA should be considered, including accessibility of target site, base-pairing energy, secondary structures of target mRNA and afsRNA, off-target effects of afsRNA, and reaction kinetics.

We previously showed that target recognition domain 2 (TRD2) of SibC, a *cis*-encoded sRNA, requires a specialized structure to repress target *ibsC* mRNA expression [13], which functions to maintain single-strandedness of TRD2. This specialized structure was utilized as a scaffold to generate afsRNA, based on the theory that it could confer single-strandedness to target recognition antisense sequences when embedded in the RNA scaffold. The newly generated afsRNAs showed promising results in silencing the *lacZ* reporter gene [14]. Furthermore, the length of the target recognition site could be shortened to 10 nt without significant loss of efficiency. The major drawback of the Sib-derived scaffold is that its design is time consuming and laborious, since alterations other than the antisense sequence are additionally incorporated to retain scaffold structure and stability. In this respect, we designed another scaffold to allow easier embedding of antisense RNA sequences [2]. The novel scaffold includes two different stable stem-loops, specifically the P1 stem of M1 RNA at the 5'-end and the SibC terminator at the 3'-end. The P1 stem is crucial for in vivo stability of M1 RNA. However, its loop region has little constraint in length, and can therefore be modified and extended with various sequences without loss of in vivo stability. P1 and terminator stems were tethered to the 5' and 3' ends of a defined antisense sequence, respectively, to generate afsRNAs having two stem-loops as a scaffold, and designated ARdSL RNA (antisense RNA in double-stem-loop scaffold). This type of antisense sequence has less chance to base-pair with sequences of the P1 or terminator stem, since both structures have intrinsic stability. Therefore, if an antisense sequence with a high degree of single-strandedness is selected, this feature would be maintained in the afsRNA structure. Using ARdSL RNAs with 20 nt antisense sequences and the *lacZ* reporter gene, we scanned the entire *lacZ* mRNA region to examine how the location of target sites affects sRNA-mediated gene regulation [2]. The most effective gene

silencing occurred when antisense target sites were present in the translation initiation region (TIR). TIR is thought to be the most accessible site because the ribosome should be loaded, and the majority of target sites of natural sRNAs are encompassed within this region of target mRNAs.

A well-designed scaffold is essential for generating afsRNAs to effectively silence mRNA expression. Here, an efficient and versatile gene silencing method using ARdSL RNA with 20 nt antisense sequences is described. In our experiments, expression of ARdSL RNAs was controlled using isopropyl β -D-1-thiogalactopyranoside (IPTG), and target sites for specific mRNAs confined to the TIR region. ARdSLs constructed for two genes in *E. coli* successfully suppressed the levels of all tested target mRNAs, clearly indicating that our protocol can be employed as a general laboratory method to silence specific mRNAs.

2 Materials

Nuclease-free water, buffers, chemicals, microcentrifuge tubes, PCR tubes, and pipette tips were required. While we used the reagents described below successfully, various equivalent reagents are available.

2.1 Equipment

1. 37 °C shaking incubator.
2. Nucleic acid quantification: Nanodrop 1000 (ThermoScientific, USA).
3. Thermal cyclers: Exicycler™ 96 (Bioneer, Korea) for qPCR analysis, C1000 (BioRad, USA) for general PCR.
4. Gel electrophoresis kit: Mupid-2plus (TaKaRa, Japan).
5. Water bath.
6. Ice maker.
7. Benchtop centrifuge, refrigerated centrifuge.

2.2 Bacterial Cell Culture

1. 1,000× Amp stock solution: Dissolve ampicillin (Amp, 100 mg/mL) in distilled water, and sterilize by filtration through a 0.2 μ m syringe filter. Store the stock solution at -20 °C.
2. 1 M IPTG solution: Dissolve 2.38 g of IPTG in 10 mL distilled water. Sterilize by filtration through a 0.2 μ m syringe filter. Store at -20 °C.
3. LB medium: Dissolve 1.0 g NaCl, 0.5 g yeast extract, and 1.0 g tryptone in 100 mL distilled water. Sterilize via autoclaving for 15 min at 121 °C. Store at room temperature.
4. LB agar plates: Dissolve the same ingredients required for LB medium and add 1.5 g micro agar in 100 mL distilled water.

Sterilize via autoclaving for 15 min at 121 °C. Allow the solution to cool to ~50 °C and add Amp (final concentration of 100 µg/mL), if required. Mix well and pour the solution into petri dishes to ~20 mL. Store plates upside down at 4 °C.

5. 14 mL round-bottomed tubes.

2.3 Design and Preparation of Antisense Sequences to Silence Specific Gene Expression

1. Oligo annealing buffer: 100 mM NaCl, 50 mM NaCl, and 10 mM Tris-HCl, pH 8.0.
2. T4 polynucleotide kinase.
3. 10× T4 ligase buffer: 500 mM Tris-HCl (pH 7.5), 100 mM MgCl₂, 100 mM DTT, 10 mM ATP, 250 µg/µL bovine serum albumin (BSA).

2.4 Construction of *afsRNA* Expression Plasmids

1. Spin column-based plasmid DNA purification kit (Intron Biotechnology, Korea, 17096, or another manufacturer).
2. *Sma*I restriction enzyme and 10× restriction enzyme buffer (Promega, USA, R6121, or another manufacturer).
3. Phosphatase: Antarctic phosphatase (New England BioLabs) (*see Note 1*).
4. Sterilized scalpels.
5. Spin column-based fragment DNA purification kit (Intron Biotechnology, Korea, 17288, or another manufacturer).
6. T4 DNA ligase.
7. 2× rapid ligation buffer: 60 mM Tris-HCl (pH 7.8), 20 mM MgCl₂, 20 mM DTT, 2 mM ATP, and 10 % polyethylene glycol (PEG).
8. Equivalent competent cells: Chemically competent DH5α *E. coli* cells (*see Note 2*).

2.5 Evaluation of the Knockdown Efficiency of *afsRNA*

RNase-free conditions must be maintained for RNA work. Nuclease contamination may lead to degradation of RNA, rendering it useless.

1. 0.01 M NaCl (dissolved in distilled water and filter-sterilized with a 0.2 µm pore).
2. 0.03 M CaCl₂ (dissolved in distilled water and filter-sterilized with a 0.2 µm pore).
3. RNA protection reagent: RNAProtect Bacteria Reagent (Qiagen, Germany) or an equivalent reagent.
4. Spin-column-based RNA purification kit: RNeasy mini kit (Qiagen, Germany) or another manufacturer.
5. Lysozyme solution: Dissolve lysozyme at a final concentration of 1 mg/mL in TE buffer (10 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0). Store at -20 °C.

6. β -Mercaptoethanol.
7. DNase I: Turbo DNA-free™ DNase Kit (Ambion, USA) or another manufacturer.
8. Reverse transcriptase: TOPscript™ RT DryMIX (Enzynomics, Korea) or another manufacturer.
9. Random hexamer (dN₆).
10. qPCR premix: TOPreal™ qPCR 2× PreMIX (Enzynomics, Korea) or another manufacturer.

3 Methods

3.1 Design and Preparation of Antisense Sequences for Embedding in an RNA Scaffold

3.1.1 Consideration of Accessible Regions on Target mRNA

Accessibility of target sites and single-strandedness of antisense sequences are critical for the effective silencing of specific gene expression (*see Note 3*). Generally, TIRs are thought to be the most accessible site on mRNAs, since these regions should be ready to accommodate ribosomes. The TIR typically includes the Shine-Dalgarno (SD) sequence and the start codon, AUG. The ribosome-binding site of *lacZ* mRNA, assessed using RNA footprinting, spans positions -14 to $+21$ relative to the start codon ($+1$ is “A” of the ATG codon) [15]. However, target regions may not be equally effective, even in the TIR, since effectiveness depends on the structural and sequence contexts of TIRs of target mRNAs [2].

We recommend preparing three 20 nt antisense sequences spanning positions -25 to -6 , -20 to -1 , and -15 to $+5$, respectively, and utilizing the most effective sequence among these that induces gene silencing (Fig. 1) (*see Note 4*).

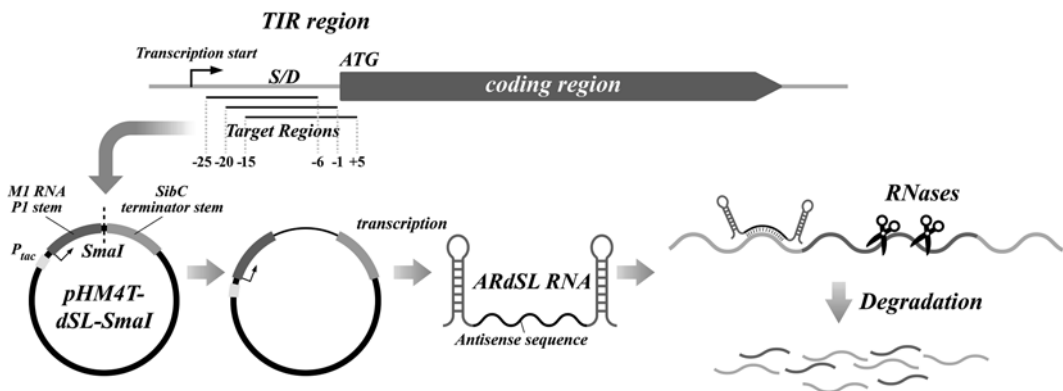


Fig. 1 Schematic view of gene silencing mediated by artificial small regulatory RNAs (afsRNAs). Schematic illustration of interactions between ARdSL RNAs (antisense RNA in double-stem-loop scaffold) used as afsRNA and target mRNA. The Shine-Dalgarno sequence (S/D), translation initiation region (TIR region), and target regions within a specific gene are indicated. Three target regions (positions -25 through -6 , -20 through -1 , and -15 through $+5$ relative to the start codon) were tested. The target recognition sequences are embedded between two stem-loop structures in ARdSL RNAs, which are induced with IPTG in cells containing pHM4T-dSL-SmaI-derived RNA expression plasmids. Interactions between ARdSL RNAs and mRNA led to gene silencing

3.1.2 Synthesis of Oligonucleotides and Annealing

1. Design three antisense oligonucleotides (oligos) complementary to positions –25 through –6, –20 through –1, and –15 through +5 of a specific mRNA.
2. Synthesize or purchase the sense and antisense pairs of oligos with lengths of 20 nt.
3. Dissolve the oligos in nuclease-free water to a final concentration of 100 pmol/μL.
4. Mix 1 μL of each sense and antisense primer with 48 μL of oligo annealing buffer.
5. Incubate the mixtures for 5 min at 95 °C and slowly cool to 30 °C or reduce the temperature from 95 to 10 °C at intervals of 5 °C for 5 min using a thermal cycler.
6. Store the annealed products at –20 °C.

3.1.3 Phosphorylation of Annealed Products

1. Make up the following mixture:

Annealed oligos	10 μL (20 pmol)
T4 Polynucleotide kinase	1 μL (10 U)
10× T4 DNA ligase buffer	5 μL
Nuclease-free water	34 μL
Total	50 μL

2. Incubate the mixture at 37 °C for 30 min (*see* **Notes 5** and **6**).
3. Store at –20 °C.

3.2 Construction of afsRNA Expression Plasmids

We used the plasmid vector, pHM4T-dSL-*Sma*I, to generate a stable double-stem-loop RNA scaffold. The vector is designed for easy cloning and IPTG-inducible expression of afsRNA [16]. Following insertion of an antisense sequence into the vector through the *Sma*I site (*see* **Note 7**), afsRNAs carrying antisense RNA embedded between the two stem-loop structures can be expressed via IPTG induction (Fig. 1).

3.2.1 Preparation of Linearized and Dephosphorylated Vector DNA

1. Prepare pHM4T-dSL-*Sma*I vector using a plasmid DNA purification kit, according to the manufacturer's specifications.
2. Digest ~3 μg of vector DNA with ten times excess unit of *Sma*I restriction enzyme (30 U) (*see* **Note 8**). Combine the following reagents:

pHM4T-dSL- <i>Sma</i> I	50 μL (~3 μg)
10× restriction enzyme buffer	10 μL
Nuclease-free water	37 μL
<i>Sma</i> I restriction enzyme (10 U/μL)	3 μL (30 U)
Total	100 μL

3. Incubate the mixture at 25 °C for 4–6 h and inactivate the restriction enzyme via denaturation at 65 °C for 20 min (*see Note 9*).
4. Add 10 µL of 10× Antarctic phosphatase reaction buffer (1/10th the reaction mixture volume) to the mixture (you do not need to consider preexisting restriction enzyme and buffer in the reaction mixture).
5. Add 1 µL of Antarctic phosphatase (5 U) for dephosphorylation of 1–5 µg DNA.
6. Mix well and incubate in a 37 °C water bath for 25 min.
7. Heat the mixture at 65 °C for 10 min to inactivate phosphatase.
8. Subject the reaction mixture to electrophoresis on a 1 % agarose gel and excise the linearized and dephosphorylated vector DNA from the gel with a sterilized scalpel.
9. Purify the cleaved vector DNA using a commercial spin column-based gel extraction kit, according to the manufacturer's specifications. Typically, 30–50 µL nuclease-free water is used for the final elution step. The final concentration of vector DNA should be >10 ng/µL.
10. Measure the concentration and purity of DNA using a spectrophotometer.

3.2.2 Ligation and Transformation

1. Mix linearized vector DNA and annealed oligos at a molar ratio of 1:3 to 1:6. The total volume should be <4 µL.
2. Add 5 µL 2× rapid ligation buffer, 1 µL T4 ligase, and nuclease-free water up to 10 µL (*see Notes 10 and 11*).
3. Incubate the mixture at 4 °C for >4 h.
4. Add 1–2 µL of the ligation mixture directly to 50 µL of chemically competent DH5α *E. coli* cells.
5. Leave on ice for 20 min and apply heat shock for 1 min in a 42 °C water bath, followed by incubation on ice for 2 min.
6. Add 1 mL of LB medium and incubate for 1 h in a 37 °C shaking incubator.
7. Plate 300 µL of cells on LB/Amp agar plates and incubate overnight in a 37 °C incubator.

3.2.3 Preparation of *afsRNA* Expression Plasmid

1. Streak a transformed colony on fresh LB/Amp plates for further purification (*see Note 12*).
2. Inoculate a single colony into 3 mL LB medium containing 100 µg/mL Amp and grow overnight at 37 °C with shaking.
3. Harvest cells and prepare the recombinant plasmid using a plasmid DNA purification kit, according to the manufacturer's specifications.

4. Confirm the desired recombinant plasmid via sequencing analysis.
5. Measure the DNA concentration using NanoDrop 1000 and store at -20°C for further use.

3.3 Evaluation of the Knockdown Efficiency of Designed afsRNA

We assessed the utility of our protocol as a routine gene silencing method by applying to two endogenous mRNA targets in *E. coli* MG1665, which are expressed as monocistronic mRNA (Fig. 2). In general, all samples containing RNA must be incubated on ice, unless otherwise specified.

3.3.1 Preparation of afsRNA-Expressing *E. coli*

1. Dilute 30 μL of overnight cultured *E. coli* MG1665 cells (or other strains to be tested for gene silencing) into 3 mL LB/Amp medium (1:100 dilution) and grow in a 37°C shaking incubator until $\text{OD}_{600} \sim 0.5$ (see **Note 13**).
2. Harvest 3 mL of bacterial culture via centrifugation at $5,000 \times g$ for 2 min at 4°C and discard the supernatant. After this step, cells must be stored in ice.
3. Add ice-cold 0.01 M NaCl (500 μL) to harvested cells and resuspend via pipetting. Ensure that all pellets have disappeared.
4. Centrifuge at $5,000 \times g$ for 2 min at 4°C and discard the supernatant.
5. Add 0.03 M CaCl_2 (500 μL) to cells and incubate the tube on ice for 20 min.
6. Centrifuge at $5,000 \times g$ for 2 min at 4°C and discard the supernatant.
7. Add 0.03 M CaCl_2 (100 μL) and mix well by pipetting until all the pellets have disappeared.
8. Add 10–100 ng of recombinant afsRNA expression plasmid DNA or the plasmid vector, pHM4T-dSL-*Sma*I, as the control cell group. Incubate for 10 min on ice (see **Note 14**).
9. Apply heat shock at 42°C for 1 min and leave on ice for 2 min.
10. Add 1 mL of LB medium to each tube and plate 300 μL of the sample on LB/Amp agar plates.
11. Incubate overnight at 37°C . Turn the plates upside down during incubation.
12. Streak one of the transformed colonies on fresh LB/Amp plates for colony purification.
13. Inoculate a single colony and make freezer stocks for later use.

3.3.2 Total RNA Preparation

1. Inoculate a single colony of plasmid-containing *E. coli* cells (recombinant afsRNA expression plasmid or empty vector, pHM4T-dSL-*Sma*I, as the control) into 3 mL LB/Amp medium overnight in a 37°C shaking incubator.

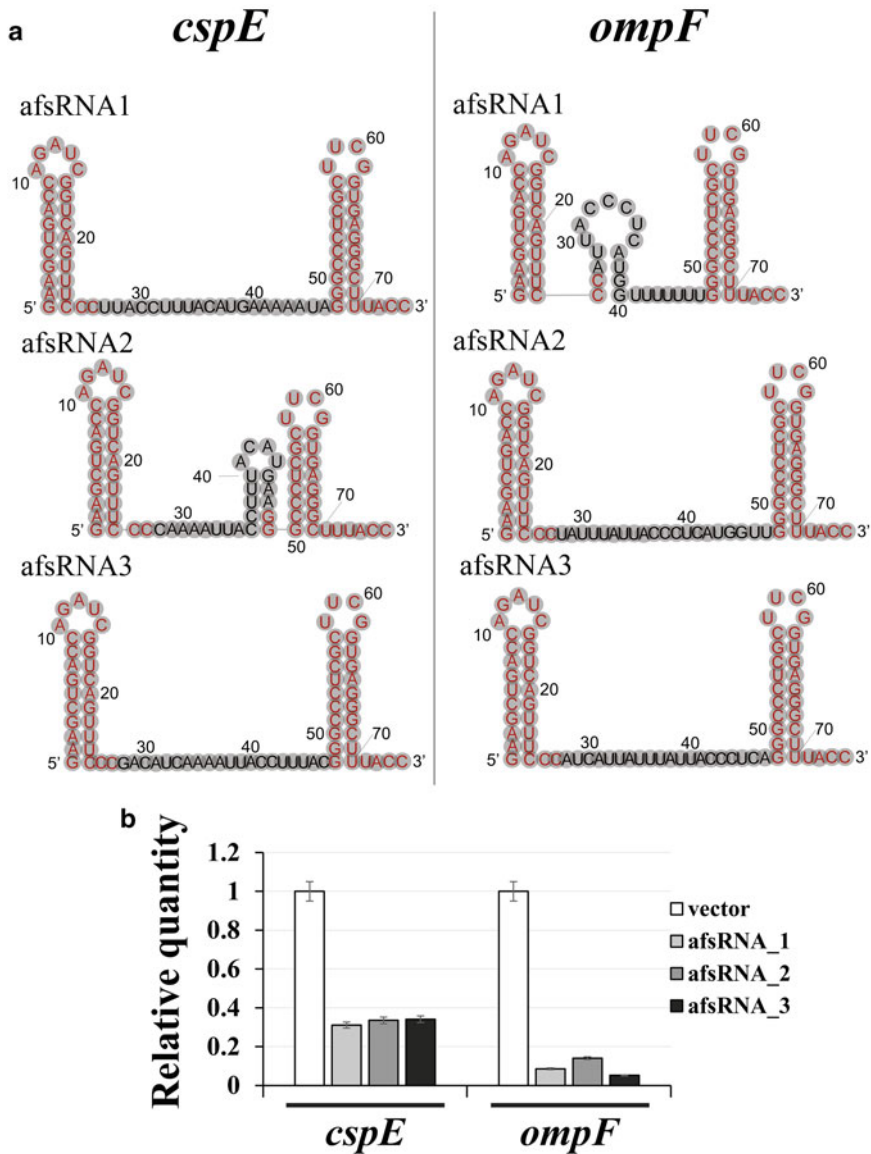


Fig. 2 Gene silencing effects of afsRNA. (a) Prediction of the secondary structure of afsRNAs. For each mRNA, three afsRNAs recognizing different target regions were prepared. Mfold program was used to calculate the lowest energy of formation and predict the most appropriate model. Two distinct stem-loops are shown in red. (b) Fold changes of *cspE* and *ompF* mRNA level relative to empty vector control following afsRNA induction. The expression level of each mRNA was determined by qRT-PCR and the data are averages of triplicate experiments, and the error bars indicate standard deviations. For qRT-PCR, the following primers were used: 5'-GTCCAAAGGATTCGGTTTCA-3' and 5'-CAGAGCGATTACGTTTGAG-3' for *cspE*, 5'-AGGCTTTGGTATCGTTGGTG-3' and 5'-TGCGCAACTAACAGAACGTC-3' for *ompF*, and 5'-AACGCGAAGAACCTTAC-3' and 5'-CGGTGTGTACAAGGCCCGGG-3' for *rrsA* (16S RNA) (color figure online)

2. Dilute overnight-cultured cells (1:100) in LB/Amp medium and grow for 3–4 h ($OD_{600} \approx 1.0$). Add 1 mM IPTG (final concentration) to induce afsRNA expression for gene silencing.
3. Stabilize RNA of harvested cell cultures using two volumes of RNeasyprotect Bacteria Reagent.
4. Mix well by vortexing for 5 s and incubate for 5 min at RT.
5. Centrifuge for 10 min at $5,000 \times g$. Discard the supernatant fractions by gently pouring and remove residual supernatant by dabbing the inverted tube onto a paper towel, since the centrifuged pellet is small and transparent.
6. Add 200 μ L of 1 mg/mL lysozyme solution for disruption of the cell wall and incubate at RT for 10 min with 10-s occasional vortexing.
7. Prepare total RNA using the RNeasy mini kit according to the manufacturer's specifications (*see* **Note 15**).
8. Use 50 μ L nuclease-free water for the final elution step. Typically, the final concentration of purified total RNA is >500 ng/ μ L.
9. Store the purified RNA at -70 °C.

3.3.3 DNase Treatment

1. Make up the following mixture in a PCR tube, using the Turbo DNA-free™ DNase Kit to remove contaminated genomic DNA:

Purified total RNA + nuclease-free water	10 μ g in 50 μ L
10 $\times$ Turbo DNase buffer	5 μ L
Turbo DNase I (2 U/ μ L)	1 μ L (2 U)
Total	66 μ L

2. Incubate at 37 °C for 30 min.
3. Add 2 U of DNase I again and incubate at 37 °C for 30 min.
4. Add 10 μ L (0.2 volumes) of inactivation slurry containing the inactivation reagent of DNase I. Since the slurry tends to sink, vortex well before adding.
5. Tap the mixture and incubate at RT for 10 min. During incubation, vortex for 5 s at least every 2 min.
6. Centrifuge at $10,000 \times g$ for 1.5 min. Collect 50 μ L of the supernatant.
7. Store at -70 °C.

3.3.4 cDNA Synthesis

1. Make up the following mixture to synthesize the first strand of cDNA (*see* **Note 16**):

DNase-treated total RNA	4 µL (~800 ng)
Nuclease-free water	14 µL
Random hexamer (dN ₆)	2 µL (50 pmol)
TOPscript™ RT DryMIX	1 tube
Total	20 µL

2. Mix well by vortexing.
3. In the PCR machine, incubate the mixture at 50 °C for 60 min and heat-inactivate reverse transcriptase at 95 °C for 5 min.
4. Store the cDNA at –20 °C until the real-time PCR reaction.

3.3.5 qPCR Analysis of Gene Expression Suppression by *afsRNAs*

1. Prepare the appropriate primers for qPCR analysis of each target gene (*see* **Note 17**).
2. Add 1 µL of DNase I (2 U/µL) in 1 mL of qPCR premix to remove contaminated genomic DNA and incubate at 37 °C for 30 min (*see* **Note 18**).
3. Add 1 µL of DNase I again and incubate at 37 °C for 30 min.
4. Inactivate the DNase I reaction at 75 °C for 15 min and store on ice.
5. Prepare the quantitative real-time PCR mixture (*see* **Note 19**):

Gene-specific primers	1 µL each (10 pmol)
Template cDNA	2 µL
TOPreal™ qPCR 2× PreMIX	25 µL
Nuclease-free water	21 µL
Total	50 µL

6. Prepare the following controls:

- Empty pHM4T-dSL-*SmaI* control for comparing the quantity of target mRNAs:

Gene-specific primers	1 µL each (10 pmol)
Template cDNA of pHM4T-dSL- <i>SmaI</i> control	2 µL
TOPreal™ qPCR 2× PreMIX	25 µL
Nuclease-free water	21 µL
Total	50 µL

- 16S rRNA (*rrsA*) for normalization (*see* **Note 20**):

<i>rrsA</i> sense, antisense primers	1 µL each (10 pmol)
Template cDNA	2 µL
TOPreal™ qPCR 2× PreMIX	25 µL
Nuclease-free water	21 µL
Total	50 µL

- No reverse transcription control (*see* **Note 21**):

Gene-specific primers or <i>rrsA</i> primers	1 µL each (10 pmol)
DNase-treated RNA mixture of pHM4T-dSL- <i>SmaI</i> control or target afsRNAs (<i>see</i> Note 22)	2 µL
TOPreal™ qPCR 2× PreMIX	25 µL
Nuclease-free water	21 µL
Total	50 µL

- No-template control (*see* **Note 23**):

Gene-specific primers or <i>rrsA</i> primers	1 µL each (10 pmol)
TOPreal™ qPCR 2× PreMIX	25 µL
Nuclease-free water	23 µL
Total	50 µL

- Seal the plate with optical adhesive film.
- Mix well with a vortex mixer and spin down briefly.
- Set the tubes on a real-time PCR system and perform the following amplification reaction, according to the manufacturer's specifications:

1 cycle	95 °C	10 min
40 cycles	95 °C	30 s
	57–60 °C	30 s
	72 °C	20 s (depending on the product size)
	Scan	
1 cycle	25 °C	10 min (or melting step at 65–95 °C)

- Following the PCR reaction, perform agarose gel electrophoresis to examine specific amplification (*see* **Note 24**).
- Analyze the data by using the “ddC_T method,” according to the manufacturer's specifications (Fig. 2) (*see* **Note 22**).

4 Notes

1. Antarctic phosphatase facilitates easier heat inactivation in 5 min at 70 °C. This property can be used to protect DNA from nonspecific degradation by the remaining activity of phosphatase.
2. A strain with the endA1 mutation, such as DH5 α , is useful for recombinant DNA work. The endA1 mutation inactivates endonucleases that degrade plasmid DNA.
3. Single-strandedness may be evaluated using secondary structure prediction programs, such as Mfold.
4. The antisense sequence could be designed to be longer or shorter than 20 nt, depending on the target mRNA. However, it is noteworthy that the length of antisense RNA can be shortened to 10 nt [14]. The 10 nt sequences occur at a frequency of one in $\sim 10^6$ (4^{10}), suggesting that these shorter antisense sequences have little chance of recognizing nonspecific target genes to lead to off-target silencing in *E. coli*. Longer base pairs may facilitate the recognition of multiple target sites, increasing the chance of potential off-target silencing. Avoid potential RNase E-dependent cleavage sites (G/A-A-U-U-U/A) in the antisense sequence that may cause rapid degradation of afsRNAs in vivo.
5. If oligos have already been modified with 5' phosphate instead of the hydroxyl end, you can skip **step 6**.
6. The reaction buffer should contain 1 mM ATP, such as T4 ligase buffer.
7. Other RNA scaffolds may be used to accommodate antisense RNA sequences [6–9, 14].
8. The total reaction volume usually varies within the range of 20–100 μ L. A large volume of water is often helpful for digestion, mainly due to dilution of potential impurities. Addition of BSA at a final concentration of 100 μ g/mL facilitates specific enzyme digestion and reduces nonspecific cutting.
9. Digestion of DNA for molecular cloning is recommended for at least 4 h.
10. The final concentration of total DNA should be 1–10 ng/ μ L. The concentration can be controlled by changing the amount of DNA or total volume of the ligation mixture.
11. 10 $\times$ T4 ligase buffer is usable, but we observed more efficient ligation with 2 $\times$ rapid ligation buffer.
12. Selected colonies can be subjected to colony PCR to distinguish self-ligated vector from recombinant plasmid.

13. Growth times can be changed, depending on the strain. Higher OD₆₀₀ than 0.7 may decrease the transformation efficiency. We used *E. coli* MG1655 as a sample strain where a specific gene is silenced.
14. Excess amounts of DNA may decrease the transformation efficiency. If the plasmid concentration is higher than 100 ng/μL, DNA can be diluted.
15. The acidic hot phenol method is also acceptable. However, even a small volume of remaining phenol may inhibit subsequent enzymatic reactions. Other phenol-free RNA preparation methods for bacterial cells can be employed.
16. To optimize reverse transcription, we recommend denaturing the mixture containing nuclease-free water, template RNA, and dN₆ at 70 °C for 5 min, followed by cooling on ice.
17. The primers should have the least self-complementarity to avoid dimers, and the PCR product should be short (generally about ~100 bp).
18. DNase treatment of the *E. coli* qPCR premix is necessary, since polymerase in the premix is usually purified as recombinant proteins from *E. coli*. Additional DNase buffer may be required, depending on the composition of the qPCR premix.
19. Since qPCR is a highly sensitive technique, variability should be kept to a minimum to reduce errors. It would be convenient to prepare a master batch premix containing primers, 2× qPCR PreMIX, and nuclease-free water. At least 2 μL of cDNA is required to minimize pipetting errors.
20. The level of invariant endogenous control is used to correct for sample-to-sample variations and should not vary across the samples.
21. It is important to include no reverse transcription control to monitor remaining genomic DNA from DNase-treated RNA samples. If qPCR detects a signal from no reverse transcription control (a C_T less than 35), the RNA should be treated more thoroughly with DNase I.
22. For reverse transcriptase negative control, use 2 μL of a mixture of DNase-treated total RNA (4 μL) and nuclease-free water (16 μL).
23. If a product appeared in no-template control, it probably indicates that contaminating gDNA is present in the qPCR premix reagents.
24. Along with agarose gel electrophoresis, melting curve analysis can be used to assess PCR quality.

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